Plant Biology ISSN 1435-8603

RESEARCH PAPER

Chloroplast parameters differ in wild type and transgenic poplars overexpressing *gsh1* in the cytosol

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Keywords
Chloroplast shape; chloroplast size; glutathione; heavy metal; transgenic poplar; γ-glutamylcysteine synthetase.

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Editor
R. Mendel

Received: 5 June 2008; Accepted: 5 August 2008

doi:10.1111/j.1438-8677.2008.00146.x

ABSTRACT

Poplar mutants overexpressing the bacterial genes *gsh1* or *gsh2* encoding the enzymes of glutathione biosynthesis are among the best-characterised transgenic plants. However, this characterisation originates exclusively from laboratory studies, and the performance of these mutants under field conditions is largely unknown. Here, we report a field experiment in which the wild-type poplar hybrid *Populus tremula* × *P. alba* and a transgenic line overexpressing the bacterial gene *gsh1* encoding γ-glutamylcysteine synthetase in the cytosol were grown for 3 years at a relatively clean (control) field site and a field site contaminated with heavy metals. Aboveground biomass accumulation was slightly smaller in transgenic compared to wild-type plants; soil contamination significantly decreased biomass accumulation in both wild-type and transgenic plants by more than 40%. Chloroplasts parameters, *i.e.*, maximal diameter, projection area and perimeter, surface area and volume, surface/volume ratio and a two-dimensional form coefficient, were found to depend on plant type, leaf tissue and soil contamination. The greatest differences between wild and transgenic poplars were observed at the control site. Under these conditions, chloroplast sizes in palisade tissue of transgenic poplar significantly exceeded those of the wild type. In contrast to the wild type, palisade chloroplast volume exceeded that of spongy chloroplasts in transgenic poplars at both field sites. Chlorophyll content per chloroplast was the same in wild and transgenic poplars. Apparently, the increase in chloroplast volume was not connected to changes in the photosynthetic centres. Chloroplasts of transgenic poplar at the control site were more elongated in palisade cells and close to spherical in spongy mesophyll chloroplasts. At the contaminated site, palisade and spongy cell chloroplasts of leaves from transgenic trees and the wild type were the same shape. Transgenic poplars also had a smaller chloroplast surface/volume ratio, both at the control and the contaminated site. Chloroplast number per cell did not differ between wild and transgenic poplars at the control site. Soil contamination led to suppression of chloroplast replication in wild-type plants. From these results, we assume that overexpressing the bacterial *gsh1* gene in the cytosol interacts with processes in the chloroplast and that sequestration of heavy metal phytochelatin complexes into the vacuole may partially counteract this interaction in plants grown at heavy metal-contaminated field sites. Further experiments are required to test these assumptions.

INTRODUCTION

Poplar mutants overexpressing the bacterial genes *gsh1* or *gsh2* encoding the enzymes of glutathione biosynthesis are among the best characterised transgenic plants, and have been analysed in much detail with respect to sulphur metabolism (Rennenberg & Peuke 2005), its regulation, and cross-talk with nitrogen and carbon metabolism, as well as performance when exposed to different forms of stress (for a review see Noctor *et al.* 1998a). From these studies, transgenic poplars overexpressing the bacterial gene *gsh1* encoding γ-glutamylcysteine synthetase (γ-ECS) are thought to be good candidates for phytoremediation of soils contaminated with heavy metals (Koprivova *et al.* 2002; Bittsánszky *et al.* 2005). These poplar mutants produce enhanced amounts of glutathione (Noctor *et al.* 1996), which plays a crucial role in the detoxification of xenobiotics (Edwards & Dixon 2005) and heavy metals (Cobbett & Goldsbrough 2002). However, information on the performance of these mutants almost exclusively originates from laboratory experiments, and their performance under field conditions is largely unknown (Peuke & Rennenberg 2005a,b, 2006).

In connection with the use of these plants for practical purposes, it is important to study the influence of the genetic modification on productivity and photosynthesis. This is of particular significance for plants overexpressing *gsh1*, since increased thiol content of leaf tissues due to enhanced γ-ECS activity (Noctor *et al.* 1996; Arisi *et al.* 1997) may affect the photosynthetic apparatus. For example, overexpressing *gsh1* in the chloroplasts of tobacco plants led to structural changes in the chloroplasts and caused a decrease in the CO_2 assimilation rate (Creissen *et al.* 1999). Investigations with transgenic poplars overexpressing *gsh1* in the cytosol and with wild-type plants showed the absence of differences in a set of leaf parameters including chlorophyll, carotenoid and soluble protein levels, as well as relative water content (Noctor *et al.* 1996), plant height, and shoot and root fresh weight (Gullner *et al.* 2001). From these observations, it was concluded that photosynthetic activity was not impaired in poplars by overexpression of *gsh1* in the cytosol. However, prolonged cultivation of transgenic poplar overexpressing *gsh1* in the cytosol under controlled conditions exhibited a reduction in growth compared to the wild type (Herschbach, unpublished results).

The present investigation was performed to test, under field conditions, whether *gsh1* overexpression in the cytosol can mediate changes in growth and chloroplast parameters, as observed for tobacco plants overexpressing *gsh1* in the chloroplasts. For this purpose, we compared biomass accumulation and chloroplast shape and size in wild-type and transgenic poplars after 3 years of growth on relatively clean soil or in soil contaminated with heavy metals.

MATERIAL AND METHODS

Wild-type poplars (*Populus tremula* x *P. alba*) of the INRA female clone 717 1-B4 and a transgenic line of this clone (line ggs11: Noctor *et al.* 1996; Arisi *et al.* 1997) overexpressing the bacterial gene *gshI* encoding γ-ECS in the cytosol were grown for 3 years at a relatively clean (control) field site and a field site contaminated with heavy metals near Yekaterinburg (Sverdlovskaja oblast, Russia: 59°59′ E/58°00′ N). The soil heavy metal content at the control site was 17 ppm Cu, 32 ppm Zn, 7 ppm Pb and 0.3 ppm Cd, and at the contaminated site was 391 ppm Cu, 573 ppm Zn, 149 ppm Pb and 3.4 ppm Cd. Fully expanded leaves from the middle of the crown, exposed to the south, were collected from 10 individuals of each plant type at each field site. Leaf disks from the middle part of the leaf blade were fixed in 3.5% glutaraldehyde in 0.15 M phosphate buffer, pH 7.4. The leaf cross-sections were visualised with a light microscope (Axiostar plus, Zeiss, Germany) on a computer display and analysed by the image analysis system SIAMS (Mesoplant, Yekaterinburg, Russia). The chloroplast projection parameters studied included maximal diameter (D_{max}), projection area (A_{chl}) and perimeter (P_{chl}) in 30 replicates per leaf disk. D_{max} was determined automatically by the measurement of internal projection diameters in 64 directions. The chloroplast volume (V_{chl}) and surface area (S_{chl}) were determined by the projection method described in detail by Ivanova & Pyankov (2002): $S_{chl} = b\ A_{chl}$, $V_{chl} = A_{chl}^2/P_{chl}\ (b^3K_p)^{1/2}$, with the geometric coefficients $b = 4$ and $K_p = 0.11$. The surface/volume ratio S_{chl}/V_{chl} and the two-dimensional form coefficient $F_{2d} = P_{chl}^2/A_{chl}$ were calculated to estimate chloroplast shape from their two-dimensional projections (Ivanova & Pyankov 2002). F_{2d} does not depend on chloroplast size. The smallest value of F_{2d} was 12.56 for a spherical shape and the more the chloroplast shape differs from spherical, the higher the values obtained for F_{2d}.

Thiol content was determined as monobromobimane derivatives by reverse-phase HPLC separation with fluorimetric detection, as previously described (Strohm *et al.* 1995). For counting chloroplasts, leaf pieces were macerated in a solution containing 5% CrO_3 and 1 N HCl and the number of chloroplast per cell was counted under a light microscope in cell suspensions in 30 replicates for both palisade and spongy tissue.

The content of chlorophylls per chloroplast was determined by dividing chlorophyll content per leaf area by chloroplast number per leaf area. The pigments were extracted from leaf disks of known area with 80% acetone and measured using an SF-46 spectrophotometer (LOMO, Russia). The content of chlorophyll a+b was calculated per unit leaf area according to the equations proposed by Lichtenthaler & Wellburn (1983). The chloroplast number per leaf area was determined by multiplying chloroplast number per cell and cell number per leaf area (described in detail by Ivanova & Pyankov 2002). The number of cells per unit leaf area was

determined in a hemocytometer. Leaf pieces of known area were placed in a volumetric test tube containing 20% KOH, heated to boiling, and carefully macerated to a cell suspension; in these suspensions, cell numbers were counted with a hemocytometer. Statistical analysis was performed using a paired *t*-test.

RESULTS AND DISCUSSION

At both field sites, transgenic poplar plants accumulated slightly less aboveground biomass than the wild type; however, the differences were not statistically significant at $P < 0.05$ (Table 1). Biomass accumulation of both wild-type and transgenic plants was significantly reduced by more than 40% at the contaminated site compared to the control site (Table 1). Apparently, overexpression of *gsh1* did not result in enhanced heavy metal tolerance in the field, as also observed under controlled conditions upon Cd exposure (Koprivova *et al.* 2002). Transgenic poplars contained higher amounts of glutathione in the leaves than the wild type, both at the control and the contaminated site (Table 2). For both poplar lines, wild type and transformant, glutathione content in leaves was higher at the control compared to the contaminated site, indicating the use of glutathione for phytochelatin synthesis in the leaves of poplars growing at the contaminated site (Table 2).

The general appearance of leaves and the microscopic views of leaf cross-sections, tissue arrangement, cell layer number and chloroplast arrangement in the cell (Fig. 1) were indistinguishable between the transgenic and wild-type poplar. Chloroplast number per cell did not differ between wild-type and transgenic poplar at the control site (Fig. 2). Apparently, overexpression of the bacterial gene *gsh1* in poplar plants did not affect chloroplast division. However, soil contamination led to suppression of chloroplast replication in leaves of wild-type plants, as indicated by reduced chloroplast number per cell. At the same time, significant differences between transgenic and wild-type poplars were found in chloroplast size and shape (Fig. 2). Compared to the wild type, transgenic poplars had larger chloroplasts, and the most pronounced differences in chloroplasts sizes were found at the control site. In plants from this site, chloroplast sizes in palisade tissue of transgenic poplar significantly exceeded those of the wild type. The maximal diameter was 1.4-fold, the mean projection area twofold, and the chloroplast volume threefold higher in palisade tissue chloroplasts of the transgenic plants. These differences decreased under heavy

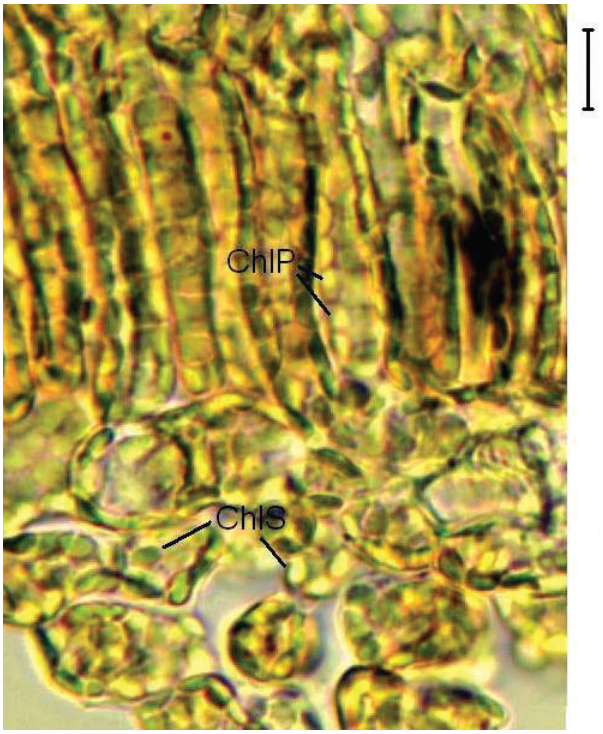

Fig. 1. Leaf sections of wild poplar grown in the control area. ChlP = chloroplasts of palisade cells; ChlS = chloroplasts of spongy mesophyll. Bars = 10 μm.

Table 1. Aboveground biomass of wild-type and transgenic poplar overexpressing the bacterial gene *gsh1* after 3 years of cultivation at a control and a heavy metal-contaminated field site.

	wild type	transgene
control site	10.66 ± 1.30[a]	8.76 ± 0.94[a]
contaminated site	5.76 ± 0.97[b]	4.51 ± 0.98[b]

Values are given in g dry weight per plant ±SD. Different letters indicate significant differences at $P < 0.05$.

Table 2. Thiol content in leaves of wild-type and transgenic poplar overexpressing the bacterial gene *gsh1* after 3 years of cultivation at a control and a heavy metal-contaminated field site.

	control site			contaminated site		
	Cys	γEC	GSH	Cys	γEC	GSH
wild type	1.7 ± 0.4[b]	0.9 ± 0.3[b]	407 ± 45[b]	4.8 ± 0.9[a]	0.1 ± 0.0[a]	223 ± 25[b]
transgene	3.5 ± 0.4[a]	2.1 ± 0.4[a]	797 ± 56[a]	2.0 ± 0.6[b]	0.5 ± 0.3[a]	493 ± 78[a]

Values are given in nmol per g fresh weight ±SD. Different letters indicate significant differences at $P < 0.05$. Cys = cysteine; γEC = γ-glutamylcysteine; GSH = glutathione; all determined as the sum of the respective oxidised and the reduced forms (Strohm *et al.* 1995).

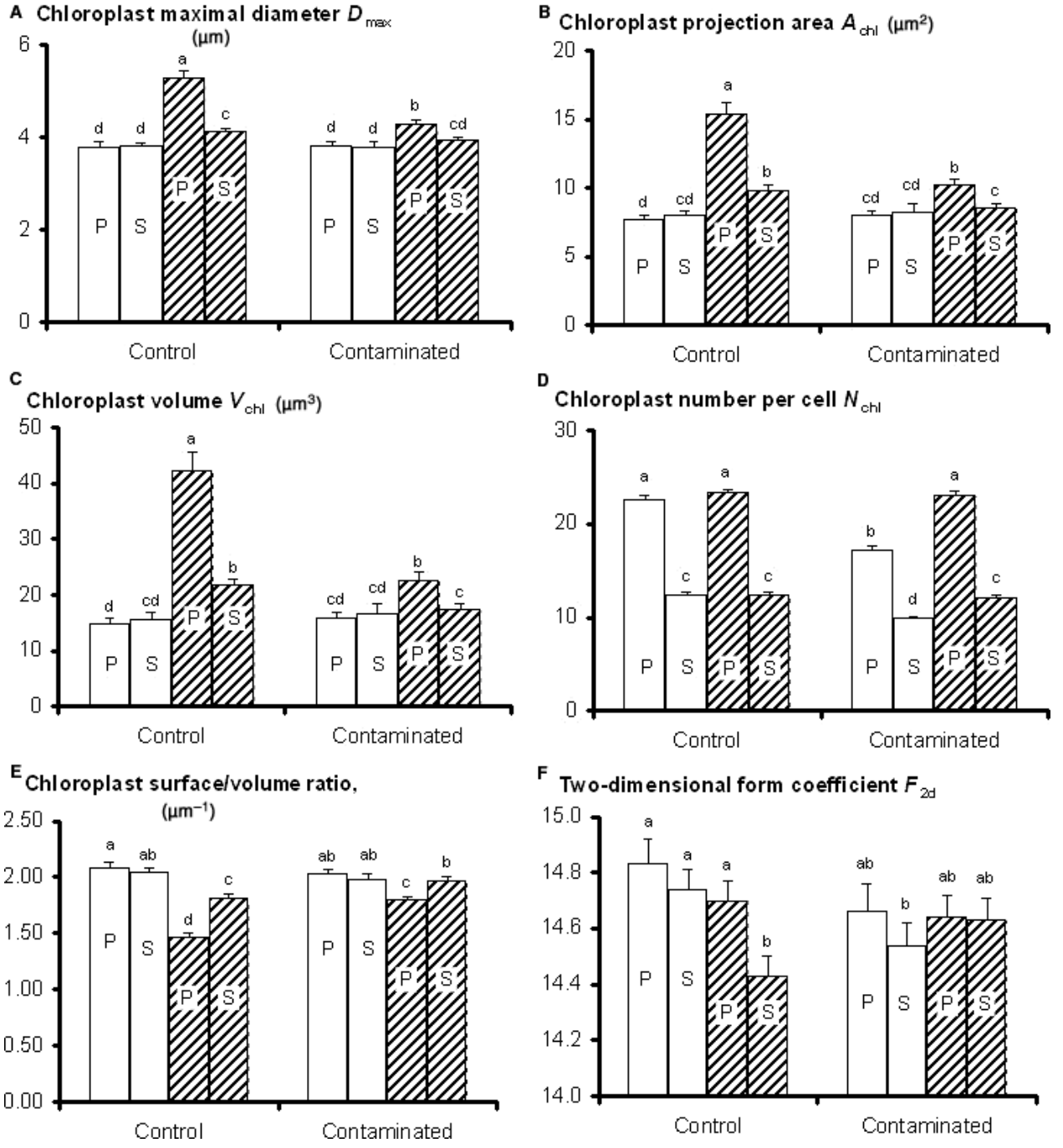

Fig. 2. Chloroplast parameters of wild (light columns) and transgenic (stroked columns) poplars at the control and contaminated sites. P = palisade tissue; S = spongy tissue. Different letters indicate significant differences at $P < 0.05$.

metals stress. Also, in spongy tissue, chloroplast size parameters increased in transgenic compared to wild-type plants. These differences disappeared in plants grown at the contaminated site. Unlike the wild-type poplars, the transformants showed significant differences between palisade and spongy tissue chloroplast size, which were highest at the control site (Fig. 2). Palisade chloroplasts were 20% larger in D_{max} than spongy tissue chloroplasts at the control site, and 10% larger at the contaminated site (Fig. 2A). As a result, palisade chloroplast volume exceeded spongy mesophyll chloroplast volume twofold at the control site and 1.3-fold at the contaminated site (Fig. 2C). Chloroplasts of transgenic poplars contained the same amount of chlorophyll as the wild type, despite

Table 3. Chlorophyll content (chlorophyll a + b) per chloroplast in leaves of wild-type and transgenic poplar overexpressing the bacterial gene *gsh1* after 3 years of cultivation at a control and a heavy metal-contaminated field site.

	control site	contaminated site
wild type	1.00 ± 0.07[a]	0.71 ± 0.05[b]
transgene	1.01 ± 0.08[a]	0.72 ± 0.05[b]

Values are given in mg per 10^9 chloroplasts ±SD. Different letters indicate significant differences at $P < 0.05$.

the differences in volume (Table 3). Therefore, overexpression of *gsh1* affects structural parameters of the chloroplasts rather than the amount of photosynthetic centres.

The estimated two-dimensional form coefficient also showed significant differences in chloroplast shape between palisade and spongy tissue chloroplasts in transgenic poplar at the control site (Fig. 2F). Palisade chloroplasts were more elongated, whereas the spongy tissue chloroplasts were spherical. In wild-type chloroplasts such a difference in shape was not observed. A difference in shape of spongy tissue chloroplasts was also not observed when plants from the contaminated site were analysed. At this site, palisade and spongy tissue chloroplasts were spherical, both in transgenic and wild-type poplar plants. The large differences between palisade and spongy tissue chloroplasts in the transformed poplars are of particular interest, because the majority of C_3 plants do not exhibit essential differences between the chloroplasts of these two types of photosynthetic tissue (Ivanova & Pyankov 2002), as also observed in the present study for wild-type poplar plants. Wild-type poplars and transformants also differed in chloroplast surface/volume ratio (Fig. 2E). Transgenic poplars had smaller values of S/V at the control site compared to the contaminated site. The S/V ratio mostly depended on the chloroplast size rather than on their shape (Fig. 3). The smallest chloroplast had the highest S/V value. In general, this ratio is directly proportional to the rate of substance transport between a body and its surroundings (Nielsen *et al.* 1996; Niklas 1997). Thus, transport between the cytosol and the chloroplasts seems to be less efficient in transgenic than in wild-type poplar.

The present results indicate changes in structural chloroplast parameters as a consequence of overexpression of bacterial *gsh1* in poplar plants, which do not result in severe changes in aboveground biomass accumulation. This result is surprising, because overexpression of *gsh1* was not directed to the chloroplasts. It may therefore be assumed that the additional glutathione produced in the transgenic line (Table 2) (Noctor *et al.* 1996; Herschbach *et al.* 2000; Koprivova *et al.* 2002; Hartmann *et al.* 2004) is exchanged with the chloroplasts and may be responsible for the structural changes observed in the chloroplasts. This conclusion is consistent with the observation of Creissen *et al.* (1999) with tobacco, where overexpression of *gsh1* in the chloroplast caused deformation of the chloroplast structure, accompanied by leaf chlorosis and necrosis. It is also supported by the finding that glutathione can quickly cross the chloroplast envelope and, therefore, glutathione pools are likely to be linked through transport across membranes (Noctor *et al.* 2002). This transport capacity may be required, *e.g.* to provide redox buffering capacity for photosynthesis (Noctor *et al.* 1998b) under conditions where glutathione is consumed by other processes. The effects of overexpression of *gsh1* in the cytosol on chloroplast structure were largely counteracted when poplars were grown at the site contaminated with heavy metals. On contaminated soil, poplar leaves contained about half of the glutathione amount found in leaves under control conditions (Table 2). At such sites, uptake of the contaminants will induce phytochelatin synthesis from glutathione as an immediate precursor (Koprivova *et al.* 2002) and the sequestration of phytochelatin–heavy metal complexes in the vacuole. Under such conditions, less glutathione may be available for exchange between the cytosol and the chloroplasts in the transgenic plants and, as a consequence, the effects of this exchange on chloroplast structure may be prevented.

In summary, after 3 years of growth in the field, overexpression of *gsh1* in the cytosol led to changes in chloroplast size and shape in transgenic poplar plants, and to significant differences between palisade and spongy tissue chloroplasts, with only minute effects on plant biomass accumulation and heavy metal tolerance. We therefore assume that (a) this overexpression can interact with processes in the chloroplast due to an exchange of the excess glutathione produced between the cytosol and the

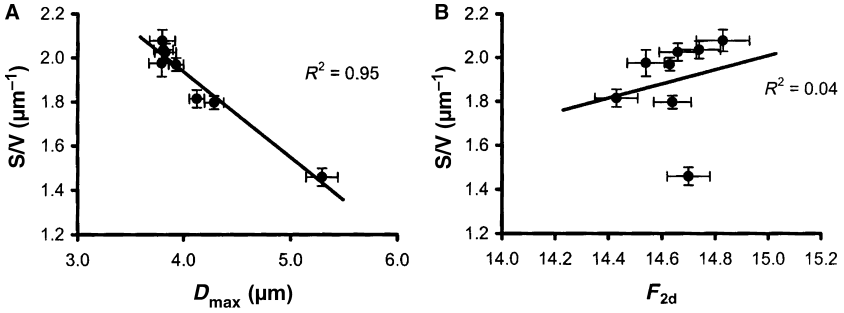

Fig. 3. Relationship between chloroplast surface/volume ratio (S/V) and maximal diameter (A) and the two-dimensional form coefficient (B).

chloroplasts, and (b) sequestration of heavy metal–phytochelatin complexes into the vacuole may partially counteract this interaction in poplar plants grown at heavy metal-contaminated sites. Further experiments are required to test these assumptions.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Bundesministerium für Bildung und Forschung (BMBF), Germany (FKZ 0312638F). We are grateful to Seregey Chrebtikov ('Green Movement' Association, Russia) for excellent technical help.

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