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Physiological and molecular responses to heavy metal stresses suggest different detoxification mechanism of *Populus deltoides* and *P. x canadensis*

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ABSTRACT

Plants have divergent defense mechanisms against the harmful effects of heavy metals present in excess in soils and groundwaters. Poplars (*Populus* spp.) are widely cultivated because of their rapid growth and high biomass production, and members of the genus are increasingly used as experimental model organisms of trees and for phytoremediation purposes. Our aim was to investigate the copper and zinc stress responses of three outstanding biomass producer bred poplar lines to identify such transcripts of genes involved in the detoxification mechanisms, which can play an important role in the protection against heavy metals. Poplar cuttings were grown hydroponically and subjected to short-term (one week) mild and sublethal copper and zinc stresses. We evaluated the effects of the applied heavy metals and the responses of plants by detecting the changes of multiple physiological and biochemical parameters. The most severe cellular oxidative damage was caused by 30 μM copper treatment, while zinc was less harmful. Analysis of stress-related transcripts revealed genotype-specific differences that are likely related to alterations in heavy metal tolerance. *P. deltoides* clones B-229 and PE 19/66 clones were clearly more effective at inducing the expression of various genes implicated in the detoxification process, such as the glutathione transferases, metallothioneins, ABC transporters, (namely *PtGSTU51, PxMT1, PdABCC2,3*), while the *P. canadensis* line M-1 accumulated more metal, resulting in greater cellular oxidative damage. Our results show that all three poplar clones are efficient in stress acclimatization, but with different molecular bases.

1. Introduction

Poplars (*Populus* spp.) are increasingly used as experimental plant material for the research of tree physiology and genetics because of their ease of cloning, transformation, and relatively small genome size. Moreover, the genome sequence of *P. trichocarpa* is known (Tuskan et al., 2006), which facilitates the use of molecular methods. Beyond the scientific importance, poplar species are highly valued by the forestry, timber and paper industries for their rapid growth, high biomass and good fiber quality. As the demand for renewable energy rises, poplars are frequently grown as bioenergy plants. In addition to their importance in timber and bioenergy production, there is increasing interest in using poplar species for phytoremediation (Marmiroli et al., 2011). Phytoremediation, however, requires considerable tolerance to contaminated soil, which frequently contains heavy metals. Understanding the cellular and molecular responses to heavy metals in poplars is therefore essential to develop new varieties and hybrids better suited for phytoremediation.

Several metals from the transition group (e.g. iron, cobalt, nickel, copper and zinc) are essential micronutrients for plants, functioning as cofactors of a number of enzymes and proteins. However, their excess in the soil and groundwater can cause growth inhibition and toxicity symptoms. Copper (Cu) is a structural element in certain metalloproteins such as Cu/Zn-superoxide dismutase, cytochrome c, ascorbate and polyphenol oxidases, which are involved in electron transport, oxidative stress response and cell wall metabolism. As a redox-active heavy metal, copper can catalyze the formation of hydroxyl radicals ($OH^{\cdot}$) from superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) in the Haber-Weiss

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reaction (Yruela, 2009), resulting in oxidative stress, lipid peroxidation, and further degradation of macromolecules (Valko et al., 2005). Zinc plays an important role in all living organisms as cofactor of hundreds of enzymes represented in all enzyme classes, zinc finger proteins, WRKY family transcription factors and 40 S ribosomal protein S27 (McCall et al., 2000; Broadley et al., 2007). In excess, zinc can inhibit plant growth and seed germination (Mrozek and Funicelli, 1982; Wang et al., 2009), change root development (Lingua et al., 2008), cause loss of membrane integrity (Stoyanova and Doncheva, 2002) or lead to cell death (Chang et al., 2005). The lack of zinc can lead to micronutrient deficiency in various crops, while excess zinc in soil is a common environmental problem in agro-ecosystems. Both excess copper and zinc can induce the formation of reactive oxygen species (ROS) and free radicals, which leads to an imbalance between antioxidant defense and accumulation of ROS (Moran et al., 1994).

In order to prevent the deleterious effects of heavy metals, plants can reduce their uptake, increase efflux pumping, chelate them in the cytosol or compartmentalize them in the vacuole (Hall, 2002). The two major groups of intracellular chelators involved in metal detoxification of plants are the phytochelatins and the metallothioneins. Phytochelatins are glutathione oligomers, their biosynthesis interacts closely with the glutathione pool, and their synthesis show heavy metal inducibility (Cobbett, 2000; Wójcik and Tukiendorf, 2004). Metallothioneins are small, cysteine-rich proteins, ubiquitous in eukaryotes and presumably widely distributed in prokaryotes as well. In *Populus* species six metallothioneins were identified and characterized by Kohler et al. (2004).

Plants have developed versatile detoxification systems to counter the toxicity of the wide variety of natural and synthetic chemicals present in the environment. These processes can also eliminate the damaged molecules originating from enhanced oxidative stress. One important detoxification mechanism is chemical modification by covalent binding to glutathione. The reaction is catalyzed by glutathione transferases (GST), a divergent group of enzymes, which are ubiquitous in all living organisms. The plant-specific tau and phi subfamilies are the most common ones in plants, but numerous smaller subfamilies are known (Dixon et al., 2002b; Dixon and Edwards, 2010). In *Populus trichocarpa*, 81 GST genes have been identified (Lan et al., 2009). While some of these genes have subsequently been characterized (Gaudet et al., 2011; Choi et al., 2013), the functions of most members of the family are still unknown. After the GST catalyzed conjugation steps, the resulting glutathione conjugates are exported from the cytosol to the vacuole by ATP-dependent tonoplast transporters (ABC transporters). While plant ABC transporters have been described in numerous species (for review see Kang et al., 2011), few experimental data are available on the role of ABC transporters in the adaptation to heavy metals (Ariani et al., 2015).

The aim of our study was to investigate the copper and zinc stress responses of three bred poplar lines, to identify such transcripts of genes involved in the detoxification mechanisms, which could play an important role in protection against xenobiotic by-products of these metals. Furthermore, we aimed to monitor the changes of a wide variety of physiological and biochemical parameters of the poplar clones under one week of heavy metal stresses, in order to provide some indication of the suitability for phytoremediation of metal contaminated sites.

2. Materials and methods

2.1. Plant material and treatments

Three poplar clones were used in these studies: *Populus deltoides* clones B-229 and PE 19/66, and *P. x canadensis* clone M-1. Woody cuttings were rooted for four weeks in tenth strength Hoagland nutrient solution (composition of undiluted nutrient solution: 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 10 μM Fe-EDTA, 10 μM H_3BO_3, 1 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 0.1 μM $CoCl_2$, pH 5.8), following two weeks in half-strength solution. Plants were grown in a growth chamber with a 12 h light:12 h dark cycle, 150 μmol m^{-2} s^{-1} photon flux density, 25 °C temperature and 55–60% relative humidity. Six-week-old poplar plants were exposed to heavy metal treatments for one week by adding copper sulphate ($CuSO_4$) or zinc sulphate ($ZnSO_4$) to the nutrient solution at a final concentration of 3 μM and 30 μM. The concentration was chosen according to the operative regulation in Hungary, in which the maximum allowed amount of copper and zinc in the ground and surface waters is limited to 200 μg L^{-1}, which is nearly equal to 3 μM for both metals. Control plants were grown in half-strength nutrient solution without additives. For all measurements the samples were taken after two and seven days of the treatments.

2.2. Analysis of heavy metal content

The copper and zinc contents of the plants were determined by inductively coupled plasma mass spectrometry (ICP-MS). Roots, shoots, and fully developed leaf material of control, 3 μM and 30 μM copper/zinc-treated poplar cuttings were harvested after one week of the treatment. Four replicates were used. After drying for 72 h at 70 °C, nitric acid (65%, w/v, Reanal, Budapest, Hungary) and H_2O_2 (30%, w/v, Reanal, Budapest, Hungary) was added to the samples, which were destroyed by microwave-assisted digestion (MarsXpress CEM, Matthews, USA) at 200 °C and 1600 W for 15 min. Cooled samples were diluted with distilled water and the element contents were determined by inductively coupled plasma mass spectrometer (Thermo Scientific XSeries II, Asheville, USA). Copper and zinc concentrations are given in μg (g dry weight)$^{-1}$.

2.3. Water status of plants

Mid-day water potential (ψ_w) of the second fully expanded leaves was measured by a pressure chamber (PMS Instrument Co., Corvallis, Oregon, USA), with 8 parallel samples from two biological replicates.

2.4. Lipid peroxidation assay

The malondialdehyde content of leaves and roots was measured with a thiobarbituric acid-reactive-substances assay, based on the formation of thiobarbituric acid-malondialdehyde conjugate by the modified method of Takeuchi et al. (1976). For the determination, 100 mg of leaf or root tissue was homogenized by a mortar and pestle with 1 mL of 0.1% trichloroacetic acid. To avoid further lipid peroxidation, 100 μL of 4% butylhydroxytoluene was added immediately to the extract. After centrifugation at 15616*g* for 20 min, 250 μL of supernatant was mixed with 1 mL 20% trichloroacetic acid containing 0.5% thiobarbituric acid and the mixture was incubated at 100 °C for 30 min. After the incubation, the volume of mixture was complete to 1.5 mL with the previous solution. The absorbance was read by an Uvikon 930 spectrophotometer (Kontron AG, Eching, Germany) at 532 nm and adjusted with non-specific absorbance at 600 nm. Malondialdehyde concentration was estimated by using an extinction coefficient $\varepsilon_{532} = 155\ mM^{-1}\ cm^{-1}$. Five samples were measured in each treatment.