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Proteomic changes in leaves of poplar exposed to both cadmium and low-temperature[☆]

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ABSTRACT

During its life span a plant has to cope with numerous and diverse constraints, which, contrary to most laboratory-trials, generally don't come one by one. Plants therefore have evolved a general adaptation syndrome, a set of molecular responses activated when exposed to various stresses. Apart from these general stress-responsive events, specific constraints result in the activation of specific responses. A major determinant in the global distribution of plant species is cold. The second constraint used in this study, cadmium exposure, is more of local importance. A poplar clone (*Populus tremula* L.) was exposed to cold, Cd and their combination and the proteome-level effects were determined. Changes in protein abundance were qualified as being either cold, Cd or general stress responsive. All treatments significantly affected plant growth but cold, singly and in combination, resulted in a complete growth arrest and effects on the F_v/F_m ratio.

Proteins involved in the methionine pathway to activated methyl groups are specifically cold-responsive, as most of the heat shock proteins and proteins with known membrane-stabilizing properties. Proteins involved in mitochondrial protein import and maturation and proteins involved in nitrogen metabolism are among the specifically Cd-responsive proteins.

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1. Introduction

During their life span, plants have to face various environmental constraints, e.g. cold, heat, drought and nutritional limitations. Additionally, plants may also have to endure various geochemical and/or anthropogenic soil or air pollutants such as ozone, fine dust or an excess of metal(loid)s. Such constraints are generally not experienced one at a time, but rather in combinations. For instance, periods of intense heat are often accompanied with drought stress, as the available water content in soils may become limiting. Likewise barren metal-contaminated soil, typical for abandoned industrial sites and mining areas is often coarse resulting in

low water retention, adding drought conditions to the presence of soil contaminants.

Although specific stress situations may induce a specific response, plants present a set of responses that are activated when growing under unfavourable conditions, independent of the constraint nature. This concept, termed general adaptation syndrome, was originally described for animal studies (Selye, 1951). It postulates that different constraints provoke similar or even identical stress coping mechanisms (Sanita di Toppi and Gabbriellini, 1999; Kranner et al., 2010). In addition, plants have a unique stress response to a combination of different constraints that cannot be simply extrapolated from single-stressor studies. When plants are exposed to a combination of stressors, the resulting response involves multiple pathways and cross-talk between different sensors and signal transduction pathways (Mittler, 2006).

The physiological and proteome-level effects of growth at low non-freezing temperature on the poplar metabolism have been previously studied (Renaut et al., 2004, 2005). Such temperature treatment induces a specific response in poplar: the survival rate is not affected but growth is almost completely arrested and tolerance to subsequent freezing increases, i.e. the plants acclimate. Cold acclimation involves metabolic changes in plant tissues, a differential regulation of genes and proteins and the

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biosynthesis of distinctive metabolic products (Kaplan et al., 2004; Theocharis et al., 2012). Similar responses have been observed in plants exposed to other abiotic stresses such as high-temperature, metal(loid) excess, drought and salinity stresses (Salekdeh et al., 2002; Ahsan et al., 2009); studies on plants exposed to a combination of stresses however are scarce. Among the most important species used to study the effects of cold on plants are rice and pea, representatives of the most important crop groups, but also the model plants *Populus sp.* and *Arabidopsis thaliana*.

Organic and inorganic soil pollutants of anthropogenic origin are widely distributed, and exposure to metal(loid) excess can affect the yield of plants. The presence of a high labile, anthropogenic cadmium (Cd) pool in soils is problematic; due to its toxicity it represents an important worldwide pollution concern. For plants and soil organisms direct toxic effects can occur while the transfer of Cd from soil to edible plant parts, the main route of exposure for humans is the consumption of Cd containing plants, makes high Cd concentrations in soil problematic for entire ecosystems. Cadmium has a high global emission, mainly stemming from industrial activities but also from the application of contaminated sludge and phosphate fertilizers on agricultural soils (Satarug et al., 2003; Zoffoli et al., 2013). Once the intercellular concentration of Cd surpasses a species-specific threshold, for maize studied by Lagriffoul et al. (1998), plants show toxicity symptoms including growth inhibition in roots and shoots and necrosis (Dias et al., 2013). Cadmium causes toxicity and damages cell metabolism in several ways. The first cause of Cd phytotoxicity is its high thiolate-affinity, an affinity that triggers the inactivation of enzymes as Cd binds to cysteine sulfhydryl-groups (Prasad, 1995; Mendoza-Cozatl et al., 2005). One of the most prominent metabolic impacts of Cd-exposure is on photosynthesis both on the light phase, by damaging the photosynthetic apparatus, but also on the dark phase, by inhibiting several of its important enzymes (Andresen and Kupper, 2013; Sanita di Toppi and Gabbriellini, 1999). Another phytotoxicity symptom of Cd-exposure is the generation of intracellular oxidative stress. Unlike other metals such as Cu and Fe, Cd does not directly produce reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($\bullet OH$) (Romero-Puertas et al., 2004). Instead it has an effect on enzymes essential for the maintenance of the cellular redox homeostasis (Cuyper et al., 2011). Its effect on the redox homeostasis is exacerbated by the chelation of Cd by glutathione resulting in the depletion of cellular glutathione pools (Clemens, 2006; Garnier et al., 2006; Márques-García et al., 2012).

One way to study stress responses in plants is differential proteomic analysis, which has been widely used to study the effects of environmental stresses, e.g. cold (Amme et al., 2006), nutritional/mineral deficiency or excess (Torabi et al., 2009; Durand et al., 2010), high light (Phee et al., 2004), salinity (Kav et al., 2004), and ozone (Bohler et al., 2010). These studies highlighted the impact of environmental constraints on the abundance of proteins involved in various metabolic pathways and indicated the increased abundance of typical stress-related proteins such as pathogenesis-related protein (PR proteins), dehydrins and heat shock proteins (Bassett et al., 2006; Durand et al., 2011).

This study focuses on proteomic changes in poplar plants exposed to sub-optimal temperatures and/or to cadmium. The effects of both environmental constraints, as single stressor, on poplar have been characterized, using a similar experimental setup (Renaut et al., 2004, 2005, 2006; Kieffer et al., 2008, 2009a, 2009b). Poplar, and more specifically *Populus trichocarpa* (Torr. & Grey) can be considered as a model tree for biological and molecular studies and its genome has been sequenced. In 2006 a draft genome was published and is currently still being annotated and refined (Yang et al., 2009). Trees from the genus *Populus* show economically important traits related to growth rate, wood properties and

paper quality which make it a potential renewable energy resource (Sannigrahi et al., 2010).

The innovative aspect of this study is to combine both factors, thereby allowing to separate cold- and cadmium-specific responses from more general stress-related responses. The discussion focuses on those effects that were qualified as being either cold or cadmium specific.

2. Materials and methods

2.1. Plant material, growth conditions and cadmium treatment

Poplar clones (*Populus tremula* L.) were multiplied in vitro in controlled growth chambers (Kieffer et al., 2008). Rooting of in vitro plants was followed by transfer and acclimation to hydroponic culture in a modified ¼-strength Hoagland's solution with a 16 h photoperiod and a light intensity of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Sylvania GroLux Fluorescent lamps). The nutrient solution was changed on a weekly basis. Plants having 22–24 leaves were divided in 2 sets; the first one as control while the nutritive solution of plants in the second set was supplemented with CdSO_4 up to a final Cd concentration of $20 \mu\text{M}$. Cadmium treatment was carried out during 56 days. At day 28 the two sets (control and Cd-treated) were again divided and half of the plants were grown for four more weeks at 4°C (chilling) compared to control conditions ($23^\circ/18^\circ\text{C}$ day/night temperature). Sampling was carried out on day 56, sampling was done in the morning and all samples were collected in a minimum of time to avoid biasing of the results. Immediately upon cutting leaves from the same foliar stage (leaf number five counted from the apex), the samples were stored in liquid nitrogen prior to starting a proteome analysis. From each condition samples from four trees were taken, thus corresponding to four biological replicates.

2.2. Growth measurements and visual symptoms

Growth of the plants during the experiment was monitored as described previously (Kieffer et al., 2008). Visual symptoms (i.e. appearances of chlorotic or necrotic spots, leaf abscission) were also recorded during the course of the experiment.

2.3. Chlorophyll fluorescence

Chlorophyll fluorescence was measured on intact leaves (4th fully expanded leaf from the top) after 30 min of dark adaptation, with a portable fluorometer Handy PEA (Hansatech; King's Lynn, UK). The fluorescence transients were measured within 5 s. F_0 is the initial fluorescence emitted by antenna Chl *a* molecules. F_m is the maximum total fluorescence value during these 5 s. $F_v = F_m - F_0$ and is the variable fluorescence. Finally the F_v/F_m ratio was calculated. The F_v/F_m ratio represents the maximum quantum yield of the primary photochemical reaction of PSII, and it has been shown that environmental stresses that affect PSII lead to a characteristic decrease in this ratio (Kocheva et al., 2004).

2.4. Soluble protein extraction and labelling

Protein extraction was performed as previously described (Kieffer et al., 2008) with slight modifications. In brief, a whole leaf (about 300 mg of FW) was crushed in liquid nitrogen and 20% trichloroacetic acid and 0.1% (w/v) DTT in ice-cold acetone was added to attain a total volume of 25 mL and kept overnight at -20°C . After centrifugation for 45 min at $35,000 \times g$ (4°C), the pellet was washed three times with ice-cold acetone/0.1% (w/v) DTT before vacuum-drying the washed pellet. Dried samples were resuspended in labelling buffer (7 M urea, 2 M

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