Environmental and Experimental Botany 75 (2012) 25–35

Contents lists available at SciVerse ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Arbuscular mycorrhizal fungi modulate the leaf transcriptome of a *Populus alba* L. clone grown on a zinc and copper-contaminated soil

Angela Cicatelli[a], Guido Lingua[b], Valeria Todeschini[b], Stefania Biondi[c], Patrizia Torrigiani[d], Stefano Castiglione[a,*]

[a] *Dipartimento di Chimica, Università di Salerno, Stecca 8, Via Ponte don Melillo, 84084 Fisciano (SA), Italy*
[b] *Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale, viale Michel 11, 15121 Alessandria, Italy*
[c] *Dipartimento di Biologia e.s., Università di Bologna, via Irnerio 42, 40126 Bologna, Italy*
[d] *Dipartimento di Colture Arboree, Università di Bologna, via Fanin 46, 40127 Bologna, Italy*

ARTICLE INFO

Article history:
Received 25 February 2011
Received in revised form 9 August 2011
Accepted 23 August 2011

Keywords:
cDNA-AFLP
Gene expression
Transcriptome analysis
Glomus mosseae
Glomus intraradices
Heavy metals
Mycorrhiza
Poplar

ABSTRACT

Significant improvement of growth associated with increased, rather than decreased, uptake of Cu and Zn has been observed in poplar plants inoculated with *Glomus* spp. as compared with non-mycorrhizal plants. The beneficial effect exerted by these arbuscular mycorrhizal fungi (AMF) is likely to be controlled by specific gene expression patterns in the plant. Until now, however, very little is known about the transcriptional changes which occur in response to heavy metals (HMs) in mycorrhizal vs. non-mycorrhizal poplar plants. In order to identify such HM- and/or AMF-induced changes in leaves of white poplar (*Populus alba* L.) plants grown, in the greenhouse, on Cu- and Zn-polluted soil, the cDNA-Amplified Fragment Length Polymorphism (AFLP) approach was adopted, resulting in the identification of a number of new differentially regulated genes. Transcript derived fragments (TDFs) mostly belonged to stress-related functional categories of defence and secondary metabolism. Genes belonging to different functional categories, plus other genes known to be related to HM stress (metallothioneins, phytochelatin synthase, glutathione synthase, arginine decarboxylase), were analysed by quantitative (q)RT-PCR. Transcript levels were generally down-regulated, or unaffected, in polluted soil compared with controls, the main exceptions being phytochelatin synthase and clathrin, and strongly up-regulated in the presence of AMF, especially *Glomus mosseae*.

1. Introduction

Heavy metals (HMs), broadly employed in several industrial processes, inevitably contribute to environmental (air, water and soil) pollution. Hence HM pollution has become a major area of concern worldwide (Pilon-Smits, 2005). Alternatively to the traditional expensive and often unsustainable clean-up methods that deal with HM pollution, phytoremediation could be exploited as a cost-effective and less disruptive technique. Trees have been suggested as suitable plants for phytoremediation of HM-contaminated areas mainly due to their high biomass production (Dickinson and Pulford, 2005). In particular, the Salicaceae (willows and poplars) possess several attributes that make them good candidates for phytoremediation purposes: they are fast-growing, easy to propagate, tolerant to high HM concentrations, adaptable to wetland systems, and, last but not least, they can be colonised by arbuscular and ecto-mycorrhizal fungi (Karlinski et al., 2010).

In natural ecosystems, the majority of plants forms mutualistic associations with soil AMF of the Glomeromycota (Parniske, 2008). In the symbiosis, the fungus colonises the roots, and forms differentiated arbuscules within cortical cells. The stable symbiosis increases the growth of the colonised plant through an improved nutrient supply by modifying nutrient availability, soil chemical properties, nutrient cycling, and microbial communities (Gamalero et al., 2009; Vivas et al., 2006). The AMF is likewise able to benefit plants under biotic and abiotic stress conditions (Liu et al., 2007), and recent studies have shown that colonisation of plants with AMF can alleviate HM-induced stress (Cicatelli et al., 2010; Lebeau et al., 2008; Lingua et al., 2008). However, alleviating HM toxicity by AMF colonisation can vary to a large extent, depending on the HM, its concentration in the soil, the fungal partner, and the conditions of plant growth (Hildebrandt et al., 2007). Although spontaneous colonisation can occur also on metal-contaminated soils, plant tolerance can be enhanced by inoculation with specific AMF (Cicatelli et al., 2010; Lingua et al., 2008). Consequently,

* Corresponding author. Tel.: +39 089 969548; fax: +39 089 969837.
E-mail address: scastiglione@unisa.it (S. Castiglione).

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doi:10.1016/j.envexpbot.2011.08.012

AMF-colonised plants can be used for phytoremediation of HM-contaminated areas, because while improving plant tolerance, they also stabilise soils, accumulate toxic metals in the fungal structures (phytostabilisation), reduce root-to-shoot translocation, alter metal bioavailability, or, in some cases, enhance phytoextraction (Hildebrandt et al., 2007). Although many of the basic biochemical mechanisms of metal accumulation and tolerance in plants have been characterised (Clemens, 2001; Memon and Schroder, 2009; Schutzendubel and Polle, 2002), the events underlying AMF-enhanced HM plant tolerance have only been partially elucidated (Hildebrandt et al., 2007; Ouziad et al., 2005). In particular, the understanding of the molecular mechanisms is limited, and hardly any information is available as regards poplar. Moreover, although a certain amount of data concerning the root transcriptome is available (Guerra et al., 2009; Hohnjec et al., 2005), very little is known with respect to gene expression in the aerial plant organs after AMF root colonisation (Garcia-Rodriguez et al., 2007; Ge et al., 2008), and nothing as regards mycorrhizal plants grown on HM-polluted soil.

Poplar, whose genome was the first to be sequenced amongst woody plants, is a favourable model for plant biologists to unravel the molecular basis of the responses to environmental stimuli in trees. Thus, the number of transcriptome-based approaches conducted in *Populus* is increasing rapidly, demonstrating the growing interest in expression profiling approaches (Duplessis et al., 2009). In the present study, which is an extension of the previous work by Cicatelli et al. (2010), a HM-tolerant clone of *Populus alba*, named AL35, previously selected during a field trial on a severely HM-polluted industrial site and shown to accumulate high amounts of zinc and copper (Castiglione et al., 2009), was used to further investigate the previously documented role of AMF in HM-stress alleviation. In fact, in a greenhouse study with AL35, we showed that colonisation by the AMF *Glomus mosseae* or *Glomus intraradices* could restore growth to control values in plants grown on soil collected from the same polluted site, in spite of enhanced accumulation, rather than exclusion, of HMs in mycorrhizal plants (Cicatelli et al., 2010).

Given the beneficial effects of AMF inoculation on poplar plants grown on a HM-contaminated soil, and the fact that the expression of some stress-related genes (metallothioneins, arginine decarboxylase, spermidine synthase) was modified by the fungal symbiosis (Cicatelli et al., 2010), it is expected that a broader range of genes is involved in the improved growth performance of mycorrhizal plants as compared with non-mycorrhizal ones. In the present work, we checked this hypothesis by conducting a genome-wide transcriptomic analysis by cDNA-Amplified Fragment Length Polymorphism (AFLP) of leaves of *P. alba* AL35 plants grown in pots containing HM-contaminated soil collected from an industrially polluted site, and pre-inoculated or not with *G. mosseae* or *G. intraradices*. Present results are discussed in relation to the findings reported in the previous study, and confirm, at the transcriptional level, the protective role exerted by AMF.

2. Materials and methods

2.1. Plant material

The poplar clone AL35 (*P. alba* L.) used in the present study was selected for its ability to tolerate high Zn and Cu concentration (Castiglione et al., 2009) during a field trial on a metal-polluted site (Serravalle Scrivia, AL, Italy). Twenty-cm long cuttings were collected in February from AL35 plants growing in the field, and stored at 4 °C until use.

2.2. Fungal inoculation

After overnight washing under running tap water, the poplar cuttings were put into 20 cm high plastic pots (750 mL) containing heat-sterilized (180 °C, 3 h) quartz sand (3–4 mm diameter). Pots were either inoculated with *G. mosseae* (Gerd. and Nicol.) Gerdemann and Trappe BEG 12 or *G. intraradices* (Schenck and Smith) BB-E (supplied by Biorize, Dijon, France) as previously described (Lingua et al., 2008), using inocula with a minimum of 60,000 propagules kg^{-1}, or were not inoculated (controls). Inoculum was provided at 50% (v/v) concentration around each cutting, using a 50 mL bottomless Falcon tube around the cutting. Plants were fed on alternate days with 80 mL of Long Ashton solution, modified according to Trotta et al. (1996). After 1 month, the plants were transferred into sterilised 7.5 L plastic pots containing either polluted or non-polluted autoclaved soil (see below). As previously reported, after two growth seasons mycorrhizal colonization (M%) was less than 1% in non-inoculated plants whereas it ranged from 5 to 23% without significant differences between the two fungal species and between polluted and non-polluted soil (Cicatelli et al., 2010).

2.3. Experimental design and growth conditions

The soil originating from the above-mentioned polluted site is a sandy loam (according to USDA specifications) and has the following chemical features: organic matter 2.24% d. wt; N, 0.01 d. wt; K 0.0237% d. wt; P 0.0026% d. wt; pH 6.2, with a mean soil total zinc concentration of 950 mg kg^{-1} d. wt and 1300 mg kg^{-1} d. wt of copper (Castiglione et al., 2009; Cicatelli et al., 2010). The non-polluted soil, collected from a nearby uncontaminated area, had similar features, and mean zinc and copper concentrations of 60 and 14 mg kg^{-1} d. wt, respectively. The chemical analyses were carried out by inductively coupled plasma optic emission spectrometry (ICP-OES) as described in Lingua et al. (2008).

Non-inoculated plants and plants pre-inoculated with either *G. mosseae* (Gm plants) or *G. intraradices* (Gi plants) were grown for two vegetative seasons (from March 2006 to July 2007) in pots containing polluted soil. Non-inoculated plants grown on non-polluted soil were taken as controls. Ten plants per treatment were prepared, placed in a greenhouse and automatically watered and fertilised as previously described (Cicatelli et al., 2010).

2.4. Sampling procedure

Leaf samples, representative of the entire foliage of the plant (excluding the youngest unexpanded leaves), were collected from all plants in each treatment in July 2006 during the first vegetative season (4-month old plants). The leaves from groups of 3–4 plants per treatment (from a total of 10) were pooled in order to have three biological repeats at each sampling time, frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.5. RNA extraction and cDNA synthesis

RNA was extracted twice, from all the above-cited leaf samples, in order to verify the reproducibility of the experiment and the RT-PCR results. Leaves were ground in liquid nitrogen and RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Milano, Italy). In order to avoid contamination by genomic DNA, total RNA was treated with RNase-free DNase (Qiagen, Milano, Italy) according to the manufacturer's instructions. The yield and purity of total RNA were quantified spectrophotometrically, and the integrity of nucleic acids was examined on denaturing agarose gel. From each sample, poly(A)$^+$ RNA was isolated using 60 μg of total RNA, by means of Dynabeads mRNA Purification Kit (Invitrogen, Milano,