Available online at www.sciencedirect.com
ScienceDirect
Gene 402 (2007) 68–80
GENE
www.elsevier.com/locate/gene

Phytoremediation of chromium using *Salix* species: Cloning ESTs and candidate genes involved in the Cr response

Silvia Quaggiotti [a,1], Gianni Barcaccia [b,*,1], Michela Schiavon [a], Silvia Nicolé [b], Giulio Galla [b], Virginia Rossignolo [a], Marica Soattin [c], Mario Malagoli [a]

[a] *Department of Agricultural Biotechnology, University of Padova, Campus of Agripolis — Viale dell'Università 16, 35020 Legnaro, Padova, Italy*
[b] *Department of Environmental Agronomy and Crop Science, University of Padova, Campus of Agripolis — Viale dell'Università 16, 35020 Legnaro, Padova, Italy*
[c] *Department of Animal Science, University of Padova, Campus of Agripolis — Viale dell'Università 16, 35020 Legnaro, Padova, Italy*

Received 1 March 2007; received in revised form 10 July 2007; accepted 15 July 2007
Available online 2 August 2007
Received by G. Theissen

Abstract

In this research a differential display based on the detection of cDNA-AFLP markers was used to identify candidate genes potentially involved in the regulation of the response to chromium in four different willow species (*Salix alba*, *Salix eleagnos*, *Salix fragilis* and *Salix matsudana*) chosen on the basis of their suitability in phytoremediation techniques. Our approach enabled the assay of a large set of mRNA-related fragments and increased the reliability of amplification-based transcriptome analysis. The vast majority of transcript-derived fragments were shared among samples within species and thus attributable to constitutively expressed genes. However, a number of differentially expressed mRNAs were scored in each species and a total of 68 transcripts displaying an altered expression in response to Cr were isolated and sequenced. Public database querying revealed that 44.1% and 4.4% of the cloned ESTs score significant similarity with genes encoding proteins having known or putative function, or with genes coding for unknown proteins, respectively, whereas the remaining 51.5% did not retrieve any homology. Semi-quantitative RT-PCR analysis of seven candidate genes fully confirmed the expression patterns obtained by cDNA-AFLP. Our results indicate the existence of common mechanisms of gene regulation in response to Cr, pathogen attack and senescence-mediated programmed cell death, and suggest a role for the genes isolated in the cross-talk of the signaling pathways governing the adaptation to biotic and abiotic stresses.

Keywords: *Salix* spp.; Differential display; Cr-regulated genes; Phytoremediation

1. Introduction

Chromium (Cr) is a transition element naturally occurring in crustal materials. It may exist in a series of oxidation states of which the major stable forms are the trivalent (Cr^{3+}) and the hexavalent (Cr^{6+}) (Zayed et al., 1998). The environmental behavior of Cr is a function of its oxidation state: Cr^{6+} compounds (chromates and dichromates) are strong oxidizers and highly toxic, and are much more mobile in soil/water systems than Cr^{3+} compounds, which tend to form relatively inert precipitates at near-neutral pH (Kotas and Stasicka, 2000).

Cr speciation also determines the mechanisms through which the metal is taken up by plants. The uptake of Cr^{3+} is passive, whereas the transport of Cr^{6+} across the plasma membrane seems to be mediated by an active process requiring metabolic energy provided by ATP hydrolysis (Skeffington et al., 1976). However, once inside cells Cr^{6+} is rapidly reduced to Cr^{3+} and most likely delivered to the root cell vacuoles in the form of metal-chelated compounds (Panda and Choudhury, 2005). For this reason, Cr is overall poorly translocated to aerial plant tissues.

Abbreviations: ABA, abscissic acid; ABC-transporter, ATP-binding cassette-transporter; AFLP, amplified fragment length polymorphism; ER, endoplasmic reticulum; EST, expressed sequence tag; ET, ethylene; GO, gene ontology; IEA, inferred from electronic annotation; MeJA, methyl jasmonic acid; PAGE, polyacrilamide gel electrophoresis; POT, proton-dependent oligopeptide transporter; RCA, reviewed computational analysis; ROS, reactive oxygen species; SA, salycilic acid; SNARE, soluble *N*-ethylmaleimide-sensitive factor attached protein receptor; TDF, transcript-derived fragment.

* Corresponding author. Tel.: +39 049 827 2814 (office).
E-mail address: gianni.barcaccia@unipd.it (G. Barcaccia).
[1] The first two authors equally contributed to this work.

0378-1119/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2007.07.021

There is no evidence indicating a potential biological role of Cr in plant metabolism to date (Sharma et al., 2003). Conversely, the phytotoxic effects of Cr have been studied thoroughly and are mainly related to the valence state of the metal at the time of exposure. Cr^{6+} is reported to be more toxic than Cr^{3+} to many plants (Adriano, 1986; Wetterhahn and Hamilton, 1989), and reacts with a number of reducing agents inside cells generating Cr^{3+} ions and intermediate oxidation states believed to be important in chromium genotoxicity (Micera and Dessi, 1988). However, plants exposed to either Cr^{3+} or Cr^{6+} may activate similar cellular responses such as plant growth retardation (Han et al., 2004; Shanker et al., 2005; Scoccianti et al., 2006; Castro et al., 2007), chlorosis and tissue necrosis (Dube et al., 2003; Sharma et al., 2003), decreased level of pigments (Sharma et al., 2003; Ouelhadj et al., 2006), inhibition of uncoupled electron transport (Dixit et al., 2002), ultrastructural modifications of chloroplasts and cell membranes (Choudhury and Panda, 2005), and degradation of proteins (Scoccianti et al., 2006). Additionally, Cr is implicated in the formation of reactive oxygen species (ROS) resulting in oxidative stress (Dixit et al., 2002; Pandey et al., 2005; Sinha et al., 2006).

To reduce heavy metal toxicity, plants have evolved a number of adaptive mechanisms, which include the synthesis of metallothioneins and organic acids as the main metal-binding ligands, while there are no evidences that phytochelatins are implicated in Cr tolerance (Shanker et al., 2004, Panda and Choudhury, 2005).

Chromium is broadly employed in several industrial processes including smelting, leather tanning, electroplating and mining. Such activities inevitably contribute to the Cr environmental pollution through the discharge of Cr-containing effluents in the aquatic bodies and the emissions in the atmosphere (Vajpayee et al., 2000; Shanker et al., 2005). In the last few years the number of Cr-contaminated sites has increased and hence Cr pollution has attained considerable attention being regarded as a major area of concern worldwide (Zayed and Terry, 2003). Alternatively to the traditional expensive and often unsustainable clean-up methods that deal with heavy metals, phytoremediation could be exploited as a cost-effective and less disruptive technique (Pilon-Smits, 2005). Trees have been suggested as suitable plants for phytoremediation of heavy metals contaminated areas being high yielding biomass and genetically variable organisms (Pulford and Dickinson, 2005). Particularly, species of the genus *Salix* possess several attributes that make them good candidates for phytoremediation purposes. Indeed, they are fast growing plants, excellent biomass producers, easy to propagate, tolerant to elevated heavy metals concentration, and adaptable to wetland systems (Pulford et al., 2001; Kuzovkina and Quigley, 2005; Tack et al., 2005; Mertens et al., 2006). In addition, willow trees could improve the stabilization of contaminated substrates and reduce the percolation of pollutants through the soil profile as a result of their high evaporation rate, water use efficiency and interception (Tack et al., 2005).

Most of the basic biochemical mechanisms of metal accumulation and tolerance in plants have been characterized to date (Clemens, 2001; Schutzendubel and Polle, 2002), even though the molecular events underlying their perception and the defence signal transduction have been only partially elucidated. Molecular differential screening of plants showing different ability to tolerate and accumulate metals is theoretically one of the most powerful tools for identifying key genes involved in the early signaling of Cr-stress. Two independent studies based on differential display with fluorescent RT-PCR (Minglin et al., 2005) and cDNA-AFLP (Fusco et al., 2005) were performed to characterize transcriptional regulation in response to Cd in *Brassica juncea* and a number of genes potentially involved in the Cd resistance and accumulation were identified.

This research deals with the identification of Cr-stress responsive genes possibly involved in the regulation of Cr tolerance and accumulation in four willow species (*Salix alba*, *Salix eleagnos*, *Salix fragilis* and *Salix matsudana*). The mRNA profiling by cDNA-AFLP method retrieved useful information on gene expression levels and on changes potentially related to the chromium response. A number of ESTs related to genes differentially expressed between treated plants and controls were isolated from root and leaf tissues and characterized using gene ontology vocabularies to describe them in terms of their associated biological process, cellular component and molecular function.

2. Materials and methods

2.1. Plant material and growing conditions

Rooted cuttings (20 cm long) of four willow species (*S. alba* subsp. *typica*, *S. elaeagnos*, *S. fragilis* and *S. matsudana* var. *tortuosa*) were planted in plastic pots filled with sand and

Table 1
List of primers used for AFLP and RT-PCR experiments

	*Pst*I primer	*Mse*I primer
	GACTGCGTACATGCAG	GACGATGAGTCCTGAGTAA
Gene	Forward primer	Reverse primer
Pubc	TTGGTACTGATTGGGAAGGTGGTT	TTTGGGCAGGATCAGCAGGATTT
Sui1	CTATTCCAACTGCTTTCGATCCCTT	CTATTCCAACTGCTTTCGATCCCTT
Sm#8	TAATGTCATGGAGGCCAAGGTTGTG	GCTTTGGCTGTCAGAGCCTTCAAGC
Sm#16	TCATGTTCATAGGATGGCCAAAGAAGC	TTCATAAATAAAATGGAGTGCCTTGAGAG
Se#25	TCTCCTAGCCAACCGGGAGCTATTC	GAAGGTGTGCCTCTGGAAGTACATAAATC
Sf#44	CAGGGAAATATCGTGGAGATGCAAA	ATAAGTACTGGACTTTGTTGTGGACGGC
Sf#46	CTACAAAAATTCTGCTGGACGATGAATTCC	GCCGGTTATATACTGTCTTTTTTGATGCTG
Sf#65	GCTTCAATAATGATGAGTTGGGGGGA	CGAGAAATACCCATTCTTATTGCACGC
Sa#143	CACAGCTCCAACAAGTAAATTCACAGATG	TCTTGTTCGATCAACTCCCTAAGTCTTCC