CADMIUM PHYTOREMEDINATION IN *HELIANTHUS ANNUUS*: AN APPROACH BY ENVIRONMENTAL MUTAGENESIS AND X-RAY RADIOGRAPHY

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phytoremediation, Helianthus annuus, cadmium genotoxicity, X-ray

The progressive deterioration in environmental quality and its involvement in human diseases are promoting the development of research and technology for pollution prevention and environmental remediation. Metals are primary contaminants and technologies based on physical chemical methods are available to separate or concentrate metals; most of these treatments have high cost of installation and management so useful and expensive system for the detection of elements and compounds in biological samples have been improved.

Higher plants may also play an important role in the remediation of metal polluted sites; plants with fungi and lichens are the bioaccumulators most frequently used for heavy metal monitoring. *Helianthus annuus* (sunflower) is an easily recognizable species with an important role in the world food webs, with high tolerance of metals which can provide sufficient quantities of material for repetitive sampling and analysis. Toxic heavy metals cause DNA damage, and their genotoxic effects in plants, animals and humans are most probably caused by their mutagenic effects [Knasmüller, S. et al. Detection of genotoxic effects of heavy metal contaminated soils with plant bioassays. Mutat. Res. 420, 37–48 (1998)]. Thus, evaluation of the mutagenicity of heavy metals is important in the actual environmental studies. In this work genotoxic damages caused from different concentration of cadmium have been studied in *Helianthus annuus* using micronuclei (MN) induction and DNA laddering evaluation by electrophoresis; peroxidase activity has been also determined as a marker of oxidative stress. Moreover exposition of dried leaves to X-rays has been done to detect cadmium uptake in leaves of sunflower doped with a solution of cadmium chloride.
STUDY OF TWO ARABIDOPSIS GENES MODULATED BY CADMIUM

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Myb transcription factors, A. thaliana, cadmium, ELF4, circadian clock

Heavy metals and metalloids such as Hg, As, Cd and Pb are extremely toxic, and pollution caused by these metals is a major environmental concern. These elements exert their toxicity by causing oxidative damage such as lipid peroxidation, enzyme inactivation, and DNA damage and by binding to protein sulfahydryl groups. Cadmium, in particular, is strongly phytotoxic, causes growth inhibition, and may cause plant death by interfering with important biochemical pathways. Our work is focusing on the molecular characterization of plant genes responsive to Cd treatment.

The Arabidopsis Myb59 encodes a transcriptional factor belonging to the R2R3MYB family and it is present in three splicing variants. It is under investigation because it was observed that one of the three variants (Myb59-1) is strongly induced by Cd treatment. Tobacco and Arabidopsis plants overexpressing Myb59-1 did not showed symptoms of Cd toxicity manifested with chlorosis and growth reduction in wild-type plants. In addition, the presence of this heavy metal in culture medium affected leaf morphology of wild-type plants whereas plants overexpressing Myb59-1 showed normal leaves. The effect of Myb59-1 was even more manifested in roots. In wild-type plants, it was observed the presence of many root primordia that do not develop lateral roots when grown in Cd-supplied medium, conversely, transgenic plants showed normal lateral root growth even in presence of high Cd concentration. Work is in progress to understand the role of Myb59-2 and Myb59-3 in relation to the presence of Cd and other environmental stresses.

It has been demonstrated that Cd exposure affects the circadian pattern of plasma testosterone levels in adult rats but the influence of this heavy metal on plant circadian clock has never been considered. We have study its effect on the expression of EARLY FLOWERING 4 (ELF4), a gene involved in photoperiod perception and controlling circadian rhythms and flowering time. It was observed that Cd inhibits the oscillatory expression of ELF4 when plants were maintained on daily light/dark cycle. Cd also altered the ELF4 expression pattern when plants were kept for many days under conditions of continuous light or continuous darkness. Furthermore, flowering time was monitored in transgenic Arabidopsis plants overexpressing ELF4 and treated with Cd. In wild-type plants Cd anticipates the time of flowering. The constitutive expression of ELF4 led to a delay in flowering time and a continuous plant growth, the exposure to Cd induced a further delay in flowering time and inhibition of plant growth. These data suggest that Cd exerts effects in plant circadian system which drives rhythms of many physiological processes and plant behaviour.
EXPRESSION OF *BRASSICA JUNCEA* BZIP TRANSCRIPTION FACTOR IN ARABIDOPSIS AND TOBACCO ENHANCES RESISTANCE TO AND DECREASES UPTAKE OF CADMIUM

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*Brassica juncea*, cadmium, bZIP, transcription factor

Cadmium, a non-essential heavy metal, is considered one of the major pollutants and disturbance in the uptake and distribution of macro and micronutrients in plants is also shown to be correlated with its toxicity. In view of the risk posed by Cd as an environmental pollutant, there has been interest to study in plants the mechanisms responsible for the ability either to take up toxic metals along with nutrients or to resist toxic metals and maintain homeostasis. By the AFLP-TP approach we have identified genes that exhibit modulated expression following Cd-treatment in *Brassica juncea* and several of them are under investigation. The characterization of the bZIP transcription factor *BjCdR15* showing 89 % similarity to Arabidopsis TGA3 revealed that it is induced 2 hours after Cd addition to culture medium and similar expression was observed after Ni and Pb treatments. When tobacco protoplasts were transfected with the *BjCdR15*-dsRED fusion protein, red fluorescence was readily detected in the nucleus. Tobacco and Arabidopsis plants transformed with this gene under the constitutive 35S promoter showed improved resistance to Cd measured as chlorophyll content, fresh weight and Cd uptake whereas wild-type plants grown on the same conditions were severely affected by Cd treatment. Furthermore, when wild-type plants were maintained in cadmium supplied medium they showed the formation of numerous root hairs and the inhibition of lateral root growth while plants overexpressing *BjCdR15* and grown on Cd-supplied medium showed normal root morphology. Therefore this study opens the possibility of using genes isolated from *B. juncea* to develop plants with improved resistance to and reduced uptake of Cd.
LEMNA MINOR RESPONSE TO CADMIUM ACCUMULATION

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Lemna minor, sulphate metabolism, O. sativa, cadmium, photosynthesis

The water plant Lemna minor is an angiosperm with a worldwide distribution. It has been used as an experimental model system to investigate heavy metal induced responses and to study the regulation of sulfate assimilation in higher plants. It is a free-floating plant, easy to culture in laboratory and it is reported to be an effective decontaminant of waste waters from many xenobiotics and some heavy metals such as Cd. This species takes up nutrients and all chemicals in the growth medium directly into the lower surface of its green fronds. In previous work we have investigated the factors influencing the response of sulphate metabolism to Cd and its interaction with photosynthesis. The sulphate uptake and reduction are essential to detoxify Cd but are also energetically very expensive and thus require an efficient and active photosynthesis. Since uptake and compartmentation of reduced glutathione (GSH), oxidized glutathione (GSSG), and glutathione conjugates are important for functions such as sulfur transport, resistance against biotic and abiotic stresses, and developmental processes, we are biochemically and molecularly characterising the response of Lemna minor in steady-state conditions and in response to excess Cd. Based on Oryza sativa genomic sequences we are isolating metal-uptake transporters genes, genes associated to GSH pathway and genes correlated to developmental processes in order to dissecting the molecular and morphological basis of metal hyperaccumulation.

Moreover, we are setting up new protocols for Lemna minor genetic transformation to further improve its use in phytoremediation.
PHYTOREMEDIATION AND BIOTECHNOLOGY FOR RECLAMATION OF POLYMETALLIC SOILS

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phytoextraction, heavy metals, transgenic, polyhistidine

Within the last two centuries the industrial production, mining industry and different urban activities caused environmental contamination on a large scale. Populations of hyperaccumulating plants can be found in naturally occurring metal-rich sites. These plants are not ideal for phytoremediation. They have an extraordinary tolerance for one metal but the growth rate is very low. Moreover most metal contaminated soils are enriched by more than two metals (polymetallic soils); such conditions strongly affects the efficiency of hyperaccumulators because the resistance to each of the metals is genetically regulated.

Assisted phytoextraction, is the use of high yielding crop plants that can take up relatively large amounts of metals responding to those management practices that increase the bioavailability of elements by the application of chemical agents and maximize the efficiency of metal uptake by the plant.

The possible future application of large scale phytoremediation must combine the high metal tolerance of hyperaccumulator genotypes and the high biomas yield of crops. This can be achieved by genetic manipulation of plants.

At the Department of Agriculture and Environmental Science, University of Udine two complementary approaches are under study. At the agronomic level the biomass yield and phytoextraction efficiency were observed for plants of *Helianthus annuus* and *Sorghum bicolor*, grown in a soil having the following concentrations of heavy metals: As 149, Cd 2.50, Co 29.3, Cu 801 and Zn 625 mg kg$^{-1}$. The evaluation of the potential of phytoremediation of our plants compared to other crops in terms of metal removal, was positive. *S. bicolor* performed better than *H. annuus* removing from the soil 220, 820 and 1944 g ha$^{-1}$ of respectively As, Cu and Zn.

At the biotechnological level specific genetic constructs coding for poly-histidine tags with chelating properties were designed to enhance the efficiency of assisted phytoextraction. The comparison of genetically modified lines of tobacco with control plants showed an increase of resistance and good growth in polymetallic contaminated soil. Further experiments to improve the chelating action of transgenes are in progress.
EFFECTS OF AtPCS1 OVEREXPRESSION ON CADMIUM TOLERANCE AND ACCUMULATION IN TOBACCO AND ARABIDOPSIS PLANTS

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PCS1overexpression, tobacco, arabidopsis, cadmium tolerance, cadmium accumulation

Phytochelatins (PCs) are thiol peptides involved in heavy metal tolerance and detoxification in plants. PCs are synthesized enzymatically from reduced glutathione (GSH) and the reaction is catalyzed by a transpeptidase, phytochelatin synthase (PCS). It was reported that in Arabidopsis AtPCS1 overexpression confers Cd hypersensitivity but increases Cd transport from roots to shoot (1, 2).

We overexpressed the Arabidopsis phytochelatin synthase gene (AtPCS1) in the non-accumulator plant Nicotiana tabacum. We transformed wild-type plants and plants harbouring the oncogene rolB, that induces an expansion of the root system (3). We verified cadmium tolerance and accumulation in relation to the level of PCs and glutathione. We demonstrated that overexpression of AtPCS1 increased PC content and enhanced Cd tolerance of rolB roots and of rolB and wild-type seedlings. This effect was greatly enhanced when reduced glutathione was added to the culture medium. An increased Cd accumulation was also observed in roots and shoots of seedlings and adult plants, matched by a higher production of PCs in both organs and also dependent on GSH supply. However plants overexpressing AtPCS1 showed the same ratio of Cd between roots and shoots as in wild-type plants. We conclude that overexpression of AtPCS1 in tobacco plants causes an increase in Cd tolerance and accumulation directly related to the availability of GSH. In contrast Cd translocation seems to be independent of AtPCS1 overexpression (4). Currently we are performing tolerance and accumulation experiments with arabidopsis plants overexpressing AtPCS1, at the same experimental conditions used for tobacco plants.

GENETIC MANIPULATION OF FOUR UNICELLULAR GREEN ALGAL SPECIES

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unicellular green algae, genetic transformation, biolistic, Agrobacterium tumefaciens

Unicellular green algae have been a model system for elucidating biological processes important for plants and animals and, in the recent years, there have been efforts to improve our understanding of algal molecular biology and biotechnology. The importance of transgenic techniques applied to algae is now being developed for various biotechnological applications including the synthesis of recombinant antibodies and vaccines and bioremediation of soil and water contaminated with heavy metals and organic pollutants. However, only few nuclear algal genomes can be routinely manipulated.

In this presentation we report results on nuclear transformation mediated by particle bombardment or Agrobacterium tumefaciens of four unicellular green algae species, previously tested for their ability to degrade phenols pollutants or steroids: Ankistrodesmus braunii CCAP202.7a, Scenedesmus vacuolatus SAG211-8b, Scenedesmus obliquus SAG276-1, and Selenastrum capricornutum UTEX1648 (Pinto et al., Biotechnology Letters 24: 2047-2051, 2002; Pollio et al., Phytochemistry 42: 685-688, 1996).

Preliminary in vitro resistance experiments have been made to find the minimum concentrations of the antibiotic kanamycin (kan) or glufoxinate-ammonium-based erbicide Basta useful for selection of putative transformed algal cells. The above-mentioned algal species were thus cultured in liquid or solid medium added with the selectable compound. It resulted that the concentration of kanamycin useful to discriminate putative transgenic algal cells was equal to 80 mg/l, whilst it was equal to 80 mg/l for Basta. Experiments of nuclear genetic transformation were carried out using microprojectile bombardment or co-culture with A. tumefaciens; two vectors were assayed, p35S-GUS-INT (nos-kan) and pG0229 (nos-bar).

Molecular analyses (PCR and RT-PCR) performed on putative transformed algal clones revealed the stable nuclear integration and expression of the transgenes in three species out four. Ankistrodesmus braunii, Scenedesmus vacuolatus and Scenedesmus obliquus expressed the kan gene while only Scenedesmus vacuolatus species expressed the bar gene. However, Selenastrum capricornutum did not reveal any sign of transgenes stable insertion. Frequencies of kanamycin and Basta resistant colonies were monitored and ranked between 17.2 to 19.3 x 10^-6 for co-culture experiments and between 0.3 to 3.3 x 10^-6 for microprojectile bombardment experiments. Further experiments are now in course to improve the efficiency of genes delivery and expression.