Screening and detection of biomarkers in chickpea plants exposed to chromium and cadmium

Author Details

Mather Ali Khan  
Centre for Transgenic Plant Development, Department of Biotechnology, Jamia Hamdard, New Delhi-110 062, India

Mauji Ram  
Centre for Transgenic Plant Development, Department of Biotechnology, Jamia Hamdard, New Delhi-110062, India

Prabhakar Jha  
Department of Botany, Jamia Hamdard, New Delhi - 110 062, India

M. Mobeen Ahmad  
Centre for Transgenic Plant Development, Department of Biotechnology, Jamia Hamdard, New Delhi-110062, India

Pravej Alam  
Centre for Transgenic Plant Development, Department of Biotechnology, Jamia Hamdard, New Delhi-110062, India

Kamaluddin  
Faculty of Agriculture and Regional Research Station, Wadura, Sopore, Sher-e-Kashmir University of Agriculture Science and Technology, Jammu and Kashmir, India

Athar Ali  
Centre for Transgenic Plant Development, Department of Biotechnology, Jamia Hamdard, New Delhi-110062, India

Usha Kiran  
Centre for Transgenic Plant Development, Department of Biotechnology, Jamia Hamdard, New Delhi-110062, India

M.Z. Abdin  
(Corresponding author)  
Centre for Transgenic Plant Development, Department of Biotechnology, Jamia Hamdard, New Delhi-110062, India  
e-mail: mzabdin@rediffmail.com

Abstract

A broad screening protocol, covering the most general phytochemical groups of compounds, was developed on the basis of high performance thin layer chromatography (HPTLC). A total of six TLC systems, comprising three derivatization reagents, two stationary phases and two mobile phases, were included. The screening method was applied for the identification of biomarkers in the chickpea plant exposed to cadmium and chromium. The biomarkers were selected on the basis of significant changes (0.26-4.6 fold) in concentration levels of phytochemicals. Totally, five different amino acids, three organic acids, one sulphur containing compound and one sugar were identified as biomarkers in chickpea exposed heavy metal.

Key words  
Screening, Biomarker, Phytochemical, Chickpea, Thin layer chromatography

Introduction

Heavy metals (HMs) belong to major pollutants that are accumulated in the environment. The accumulation in the soil, however, has become a worldwide problem leading to reduced root and shoots growth, low yield production, low nutrient uptake and impaired homeostasis. Growth inhibition is a general phenomenon associated with most of heavy metals (Peralta et al., 2001; Reichman, 2002), while the tolerance limits for heavy metal toxicity are specific not only for species but also for each variety of crop plants (Liu et al., 1994; Metwally et al., 2005). The heavy metals are frequently accumulated by agriculturally important crops with a significant potential to impair animal and human health (Sanita and Gabbrielli, 1999).
HMs enters the environment mainly from industrial processes, the sewage-sludge and phosphate fertilizers (Davis, 1984) and then transferred to the food chain (Wagner et al., 1993). Of all toxic heavy metals, cadmium (Cd) ranks the highest in terms of damage to plant growth and human health. Daily consumption of Cd-contaminated foods poses a serious risk to human health. While in plants, Cd produces alteration at various stages of physiological processes resulting growth retardation, inhibition of enzymes, and altered stomatal action (Barcelo and Poschenrieder, 1990; Siddhu et al., 2008; Neelima and Reddy, 2003). Photosynthesis is also sensitive to Cd, chlorophyll being one of the targets (Somasekharakarah et al., 1992), as well as the enzymes involved in CO₂ fixation (Greger and Ogren, 1991).

Chromium (Cr⁶⁺) is considered the most hazardous to animals and plants due to its high solubility, mobility, toxicity as well as carcinogenic and mutagenic properties (Dixit et al., 2002). It was found that occupational exposure to Cr⁶⁺ compounds lead to a variety of clinical problems. The biotoxicity of Cr⁶⁺ is largely a function of its ability to cross biological membranes, its powerful oxidizing capabilities and its interference with electron transport in respiration and photosynthesis (Losi et al., 1994).

Traditional screening methods have used to study the pharmacological effects of phytochemical compounds. Nomura et al. (2002) investigated the chemistry of phenolic compounds of licorice (Glycyrrhiza species) and screened for their estrogenic and cytotoxic activities. Koleva et al. (2002, 2003) used different methods to screen antioxidative activity of extracts from Sideritis species (Labiateae) while Couladis et al. (2003) screened Greek aromatic plants for antioxidant activity. Screening methods, using high performance liquid chromatography (HPLC) with mass spectrometer (MS) assay, also have been used to detect phytochemical compounds and phenylpropanoids in cereals (Bily et al., 2004). Raharjo and Verpoorte (2004) evaluated four different methods, of which one is gas chromatography (GC) that have been used for biomarkers in plants exposed to herbicides and pesticides.

Three groups of replicates of control and exposed plants were prepared using crushed freeze-dried plant materials. 0.3 g of plant material was extracted in three ml of 75% ethanol for 2 hr in an ice-cooled ultrasonic bath (UC-250W IMECO, India). The extracts were centrifuged in a micro centrifuge at 5000 rpm for 10 min and immediately used for analysis. 2 µl of the plant extracts were spotted on pre-coated TLC (20 X 10 cm) plates, using a semiautomatic spotter under a nitrogen stream. Plates were developed in CAMAG twin-trough chamber previously saturated with 50 ml of mobile phase for 40 min. One-dimensional TLC was performed until the solvent front was 1.0 cm from the top of the TLC-plates. Several TLC systems were used. Each TLC system is composed of a TLC plate (20 X 10 cm), a chromatographic solvent and a derivatization reagent. Three groups of replicates of control and exposed plants were used while the derivatization reagents varied between the all test systems. The stationary phases were, plate A: made of silica gel 60 on HPTLC aluminium sheets (No. 1.05547 Merck) and plate B: made of silica gel 60 on Thin Layer Chromatography (TLC) aluminium sheets (No. 1.05554 Merck). The mobile phases were: Solvent 1; consisted of 1-butanol: acetic acid: water in a ratio of 4:1:5 (upper phase after shaking for 5 min) and Solvent 2; consisted of 1-propanol: 25% ammonia/water in the ratio of 11:9. All the derivatization reagents (except for thymol at plate type A) were used in each TLC-System, and hereby 8 different combinations of the screening methods were used. The screening method was developed for quantification, as the separation was optimal for all compounds in the plant extract. Different derivatization reagents were used to detect different functional group of biomarkers. RF-values (Ganshirt, 1969) described the location of the biomarkers on the TLC-plates.

For the detection of biomarkers, the plates were photographed using CAMAG winCATS 3.00 software (Switzerland),...
Screening of biomarkers in chickpea plants exposed heavy metals

CAMAG Ultraviolet (UV) lamp (254 nm, 366 nm and white light), a CAMAG Reprostar 3 with a Canon PSG2 digital camera. CAMAG winCATS programme version 1.3.2 software was used for computer analysis. The identification and quantification of biomarkers was performed using standards, Rf-values, colour reaction in different TLC systems and UV-VIS spectra of both standard and the biomarkers from CAMAG TLC Scanner 3.00.

Results and Discussion

The phytochemical screening method was developed to provide a simple, rapid and sensitive method to detect phytochemical compounds, present in high concentration in ethanolic extracts of heavy metal exposed plants. To find the best solvents, the combination of polarity of the solvents was used according to Snyder (Snyder, 1974). The screening method was developed using ethanolic extract of chickpea plants, exposed to two heavy metals (Cd$^{2+}$ and Cr$^{6+}$). The extracts using 75% ethanol/water represented the best view of phytochemical compounds, since both partly polar and non-polar compounds were extracted. In the present study, all compounds were identified and quantified by comparing with standards. The Rf-values, colour reactions and UV-VIS spectra of these compounds and standards facilitated their identification. All the biomarkers were

<table>
<thead>
<tr>
<th>Rf-values</th>
<th>Detected compounds</th>
<th>% Variation in concentration as compared to control plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cadmium treated plants</td>
</tr>
<tr>
<td>0.75 ± 0.03</td>
<td>Isoleucine</td>
<td>(+)56.26 ± 2.98</td>
</tr>
<tr>
<td>0.70 ± 0.03</td>
<td>Tyrosine</td>
<td>(+)77.83 ± 2.30</td>
</tr>
<tr>
<td>0.57 ± 0.02</td>
<td>Alanine</td>
<td>(+)10.36 ± 0.88</td>
</tr>
<tr>
<td>0.31 ± 0.01</td>
<td>Arginine</td>
<td>(+)125.02 ± 8.30</td>
</tr>
<tr>
<td>0.38 ± 0.02</td>
<td>Lysine</td>
<td>(+)126.26 ± 8.98</td>
</tr>
<tr>
<td>0.63 ± 0.03</td>
<td>Threonine</td>
<td>(+)115.33 ± 4.22</td>
</tr>
<tr>
<td>0.50 ± 0.02</td>
<td>Valine</td>
<td>(+)41.26 ± 3.53</td>
</tr>
<tr>
<td>0.54 ± 0.02</td>
<td>Proline</td>
<td>(+)28.04 ± 1.88</td>
</tr>
</tbody>
</table>

(+): denotes increase in concentration, Each values represent mean ± SE (n=3)
selected on the basis of remarkable changes of their content level in Cd and Cr exposed plants. The stationary phases with silica gel 60 and mobile phases of 1-butanol: acetic acid: water (4:1.5) and 1-propanol: 25% ammonia (11:9) gave the best separation of the detectable phytochemical compounds after derivatization with the different reagents. The amino acids were detected as red, yellow and violet spots on a white plate in visual light using ninhydrin. All the biomarkers detected and identified in ethanolic extract of chickpea plants exposed to Cd and Cr are presented in Table 1 to 4.

Metabolites of primary metabolism can act as signal molecules. A well-known example is sucrose (Koch, 1996; Chiou and Bush, 1998; Roitsch, 1999; Smeekens, 2000; Rolland et al., 2002), whose content was found to increase in response to Cd and Cr exposure to plants. Thus, sucrose could be a candidate signalling molecule for Cd and Cr exposed plants based on accumulation in response to Cd and Cr stress. In addition to its signalling role, sucrose and other soluble sugars (maltose, Glc, and Fru) act as compatible solutes during abiotic stresses, such as cold, drought, desiccation, salt, and osmotic stress (Guy et al., 1992; Fan et al., 1993; Rizhsky et al., 2004). The biomarker proline has previously been described as a compound present at increased concentration following various types of stress exposure in plants, viz. water stress (Irigoyen et al., 1992), drought stress (Good and Zaplanchinski, 1994), heavy-metal stress (Mehta and Gaur, 1999) and NaCl stress (Lee and Liu, 1999). In our study, proline content increased by 28.04±1.88 and 26.18±1.56%, respectively, in Cd and Cr exposed plants. Arginine, lysine and threonine levels were also increased by 125.02±8.30, 126.26±8.98 and 115.33±4.22%, respectively, in Cd exposed plants after 48 hr of the treatment and, in Cr treated plants, the level of arginine (57.10±2.68%), threonine (30.61±1.34%) and valine (50.62±2.30%) were found to increased after 48hr of the treatment. The biomarkers lysine, threonine, valine, isoleucine and leucine have previously been described as compounds present at increased concentration in Apera spica-venti L. plants exposed to herbicides (Ravn et al., 2005).

Saponins and coumarins are constitutively produced in many plant species. The level of sarsasapogenin increased in Cd and Cr exposed chickpea plants. They have been suggested to constitute part of some plant defense systems. Increased levels of glutathione, citric acid, maleic acid and ascorbic acid in Cd and Cr exposed plants were also observed in this study, which may protect these plants from heavy metal induced oxidative stress (Gajewska and Sklodowska, 2005).

Abscisic acid (ABA) plays important role during many phases of the plant life cycle, including seed development and dormancy, and in plant responses to various environmental stresses. Some studies reported that the abscisic acid content increased when the plants were exposed to copper, cadmium, mercury and nickel pollution (Munzuroglu et al., 2008; Monni et al., 2001). In this study, also ABA was found to accumulate at higher levels in both Cd and Cr exposed chickpea plants.

Distinctive phytochemical differences were found between the control and heavy metal exposed samples of this crop. Out of 10 different biomarkers detected five were amino acids (arginine, isoleucine, proline, threonine and valine), three organic acids (absisic acid, ascorbic acid and maleic acid), one sulphur compound (glutathione) and one sugar (sucrose). This screening method, may possibly be used as a tool to monitored heavy metal stress not only in chickpea, but also in other crops.

Acknowledgments

Authors are highly thankful to the Incharge, Central Instrumentation Facility, Faculty of Science, Jamia Hamdard for providing instrumentation facilities to carry out the research work.

References

Irigoyen, J.J., D.W. Emerich and M. Sanchez-diaz: Water stress induced changes in concentrations of proline and total soluble sugars in...
Screening of biomarkers in chickpea plants exposed heavy metals

Lisboa, B.P.: Characterization of
Koch, K.E.: Carbohydrate-modulated gene expression in plants.
Mehta, S.K. and J.P. Gaur: Heavy-metal induced proline accumulation and
Kawerau, E. and T. Wieland: Conservation of amino acid chromatograms.
Screening of biomarkers in chickpea plants exposed heavy metals
Losi, M.E., C. Amrhein and E.T. Frankenberger: Environmental biochemistry
Metwally, A., V.I. Safronova, A.A. Belimov and K.J. Dietz: Genotypic
Liu, D., W. Jiangm, F.M. Zhaom and C. Lum: Effects of lead on root growth,
Neelima, P. and K.J. Reddy: Differential effect of cadmium and mercury on
Nomura, T., T. Fukai and T. Akaiyama: Chemistry of phenolic compounds
of licorice (Glycyrrhiza species) and their estrogenic and cytotoxic activities.

Peralta, J.R., J.L. Gardes-Torresdey, K.J. Tiemann, E. Gomez, S. Arteaga,