RESPONSE TO METAL STRESS OF *NICOTIANA LANGSDORFFII* PLANTS WILD-TYPE AND TRANSGENIC FOR GLUCOCORTICOID RAT RECEPTOR.

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ABSTRACT

Recently our findings showed that the integration into *Nicotiana langsdorffii* plants of the gene coding for the rat gluco-corticoid receptor (GR receptor) induced morpho-physiological effects in transgenic plants through the modification of their hormonal pattern. Phyto-hormones play a key role in plant response to many different biotic and abiotic stresses, a modified hormonal profile up-regulating the activation of secondary metabolites involved in the response to stress. In the present work transgenic GR plants and isogenic wild type genotypes have been exposed to metal stress treating them with 30 ppm Cadmium(II) and 50 ppm Chromium(VI). Hormonal patterns along with the changes in key response related metabolites were then carefully monitored and compared. Heavy metals absorption has been found to be lower in the case of GR plants.The transgenic plants exhibited higher values of both S-ABA and IAA phytohormones, salycilic acid and total polyphenols, chlorogenic acid and antiradical activity, as compared to the untransformed wild type plants. Both Cd and Cr treatments induced an increase in hormone concentrations and secondary metabolites only in wild type plants. These result are finally discussed proposing that the response to stress due to changes in the plant hormonal system may derive from the interaction between the GR receptor and phytosteroids, known to play a key role in plant physiology and development.

Keywords: *Nicotiana* transgenic plants, rat glucocorticoid receptor, metal stress response, phytohormones, secondary metabolites.

Introduction

Plants have developed a complex system of response processes to stress whose activation and dynamics depend on the duration and severity of the stress but also on the plant species, genotype and developmental age. Single response processes seem to be specific for each of different stress conditions as shown by the lack of an uniform transcriptome profile of plants subjected to different stress treatments such as heat and cold, drought and salinity, strong light or mechanical stress [18,19,20]. Phyto-hormones regulate integrated responses to many different biotic and abiotic stress conditions and among them, abscisic acid (ABA), jasmonic acid and ethylene, acting together with secondary messengers, such as Ca^{2+}, seem to play a key role in plant response [21]. Plant perception of stress signals is initiated by the recognition through specific receptors followed by signal transduction chains leading to the activation of the stress response network in which ABA, and other known phytohormones (auxins, cytokinins, gibberellins) play a pleiotropic regulatory key role [21].

Different stress factors affect plant growth, development and productivity. Stresses can be induced in plants by adverse environmental conditions (abiotic stress) or pathogens (biotic one). For instance, a constitutively modified endogenous hormonal background has been shown to affect the active defence response to fungal pathogens in tomato plants transgenic for the *Agrobacterium rhizogenes rol* genes [22,23] and recently, it has been reported that in *rol*-transformed cells of different *Solanaceae, Araliaceae*, *Rubiaceae*, *Vitaceae* and *Rosaceae* families a modified hormonal profile up-regulates the activation of secondary metabolites involved in the response to stress [24]. Among abiotic stresses light, water-logging, drought, heat and cold, salinity, and the presence of heavy metals can cause some of the most severe damages to plants. As reported by Weast [1], 53 out of the 90 naturally occurring elements are heavy metals, 17 out of which are of environmental concern and relevant for organisms and ecosystems [2,3]. Among these, Cd is highly phytotoxic and can cause plant death. Low mg/kg Cd concentrations have an inhibitory effect on root and shoot growth in different plant species by affecting the functionality of membranes, related enzymatic activities [7,8] and the photosynthesis rate [5,6,9], which in turn cause alterations in the uptake and distribution of macro and micro-nutrients [4-6]. The toxic effect related to chromium exposure are strongly dependent on the oxidation state and on the plant ability to accumulate Cr [10]. In fact, Cr(VI) is by far more toxic then

Cr(III) [11]. Even though Cr is poorly translocated to aerial parts, it is differently mobilized and accumulated inside tissues depending on its chemical form [12]. Cr(III) doesn't show measurable toxic effects even at several tens of mg/kg in the culture medium [13,14]. The efficiency of Cr(III) adsorption by the radical apparatus and the mobility inside the plant depend on the presence of complexing agents [12,15]. Conversely, chromate ion is able to cross the plasmatic membrane and can causes dose-related damages to the plant even if the major part of Cr(VI) is eventually reduced to the less toxic trivalent form. Cr(VI) concentration higher than 1-10 mg/kg in the culture medium produces growth inhibition, decrease in chlorophyll synthesis, reduction of photosynthesis, chlorosis inhibition of mineral absorption in various plants [11,13,14,16]. In particular, 10 mg/kg in the culture medium of *citrullus* plants produced well evident negative effects on the plant development [16], which were related to metabolic alterations either from a direct effect on enzymes or other metabolites or from the generation by this metal of reactive oxygen species which may cause oxidative stress [17].

In an earlier paper we showed that the integration into *Nicotiana langsdorffii* and *Nicotiana glauca* plants of the gene coding for the rat gluco-corticoid receptor (GR receptor) induced pleiotropic morphological and physiological effects through the modification of their hormonal pattern [25]. Namely, increasing auxin content in the first species and decreasing it in the second, thus drastically changing the citokinin/auxin ratio in both cases. Therefore the effects of the insertion seemed to be dependent from the pre-existing phyto-hormone balance of the two species [32].

The aim of this paper was to further study and compare the changes of the hormonal background pattern of the model plant (i.e., *Nicotiana langsdorfii* wilde type and transgenic for the above mentioned GR receptor) which were determined by Cr(VI) or Cd(II) exposure, thus obtaining data on the structure and dynamics of the metabolic network connected with the response to stress. Hormonal patterns, along with the changes in key response related metabolites, were carefully monitored in both genotypes. Particularly, the concentrations of some of the most relevant phytohormones, i.e. IAA (3-indole acetic acid) and S-ABA [(S)-5-(1-hydroxy-2,6,6-trimethyl-4-oxo-1-cyclohex-2-enyl)-3-methyl-penta-(2Z,4E)-dienoic acid], salycilic acid, a known inducer of active defense response, shikimic acid, a key molecule in the polyphenols synthetic pathway, and polyphenols themselves as known key secondary metabolites involved in the response of plants to environmental stress, along with metal

uptake, were measured both in wild type and transgenic plants exposed and not exposed to 30 ppm Cd(II) and 50 ppm Cr (VI).

Materials and methods

Plant material and nucleic acids extraction

Transgenic *Nicotiana langsdorffii-GR* plants are described elsewhere [25]. Briefly, leaf disk transformation experiments were performed using *Agrobacterium tumefaciens* strain LBA4404 and the binary vector pTI18 bearing the rat glucocorticoid receptor (*gr*) gene [26]. Transformants were selected using 100 mg/l kanamycin monosulfate and 500 mg/l carbenicillin. Kanamycin monosulfate (O-3-Amino-3-deoxy-alpha-D-glucopyranosyl-(1->6)-O-[6-amino-6-deoxy-alpha-D-glucopyranosyl-(1->4)]-2-deoxy-D-streptamine Monosulfate, and carbenicillin (alpha-carboxybenzylpenicillin) (antibiotics were supplied by Sigma- Aldrich, USA). Transgenic primary plants (T_0) were then grown to obtain T_1 seeds. To this purpose T_0 plants were maintained in a greenhouse under natural lighting with day length of 16 h and Temperature ranging from 18°C to 24°C±1°C. Then, transgenic plants were allowed to self-pollinate. Harvested T_1 seeds were surface sterilized with commercial Sodium hypochlorite for 20 minutes, washed three times with distilled water and placed on *Petri* dishes containing LS0 medium (Linsmaier & Skoog growth medium (LS0) was supplied by Sigma- Aldrich, USA) supplemented with 100 mg/l kanamycin. Cultures were incubated in a growth chamber at 24±1°C with a photoperiod of 16 h of light (1500 lux) and 80% relative humidity. After germination, seedlings were grown on LS0 medium for a month. Each plant was then screened for the presence and the expression of *gr* gene and multiplied by cutting internodes containing stem fragments, for further analyses.

Genomic DNA was isolated from young leaves of each T_1 plant and from *N. langsdorffii* not transformed by the use of the commercially available NucleoSpin® Plant II kit (Macherey-Nagel, M-Medical, Italy). Total RNA was isolated with the Plant RNA extraction kit (Macherey-Nagel, M-Medical, Italy) following instructions of the protocol supplied. The extracted nucleic acids were then respectively used for PCR and RT-PCR amplification of the *gr* gene as described in literature [25].

Plant exposure to Cr(VI) and Cd(II).

The concentration of Cr and Cd for the induction of metal stress in *Nicotiana* plants was selected on the basis of preliminary experiments to test the lethal-dosage 50 (LD50) on survival and callus formation capacity of leaf tissues of wild type *N. langsdorffii* plants *in vitro* grown. The standard working solutions were prepared from Potassium dichromate ($K_2Cr_2O_7$) and Cadmium sulphate ($CdSO_4$) concentrated standard solutions (Merck Titrisol). Working solution were filter sterilised by 0.22 μm Millipore membrane filter and added to the LS0 medium at different concentrations. Thirty leaf disks with a diameter of 12 mm were placed in Petri dishes containing fresh sterile medium supplemented with 0.4 mg/l of 2,4-D (2,4-dichlorophenoxyacetic acid, supplied by Sigma- Aldrich, USA) and increasing concentrations of chromium (0, 25, 50, 75 ppm) and cadmium (0, 10, 30, 50 ppm) and incubated in the growth chamber at 24±1°C. Survival efficiency and callus formation capacity of explants, expressed respectively as the percentage of green and callus forming explants against control, were determined at 15 days intervals for one month. Experiments were carried out in triplicate. Moreover, to evaluate the effect of Cr and Cd on the *in vitro Nicotiana* plant growth, five plant samples for each metal were incubated in Wavin containers (LAB Associates BV, the Netherland) with LS0 medium containing that metal at the selected concentration level. Leaf biomass was also determined before and after freeze-drying treatment for all the investigated theses.

Sample analysis (see Supplementary information for details)

Total Cr and Cd concentrations were determined in a freeze-dried and homogenate plant sample aliquot of 300 mg which was submitted to acid digestion in a microwave oven (Milestone MLS-1200 Mega microwave laboratory unit). The solutions was analysed by Inductively Coupled Plasma-Quadrupole Mass Spectrometry (ICP-QMS, Agilent 7500) equipped with a V-groove nebulizer and a cooled spray chamber (2°C).

Abscisic acid (ABA) and 3-indoleacetic acid (IAA) were determined in a freeze-dried and homogenate plant sample aliquot of 1.5 g by cold extraction (-28°C) with methanol/water/formic acid mixture (75:20:5 v/v). The final extract was analysed by a Perkin Elmer 200 liquid chromatograph

coupled with an Analytical Biosystem Sciex mod API 4000 mass spectrometer, with triple quadrupole and turbo spray ion source.

Shikimic acid (SCI) and salycilic Acid (SAL) were extracted from a 0.1 g aliquot of a freeze-dried plant sample with 0.1 M HCl and methanol 99.9:0.1 mixture. The final extract was analyzed by a Agilent series 4100 liquid chromatograph coupled with an Analytical Biosystem Sciex mod. API 4000 mass spectrometer, with triple quadrupole and turbo spray ion source.

Selected monomeric polyphenols, Chlorogenic acid, (+)-catechin, caffeic acid, gallic acid, p-coumaric acid, scopoletin, rutin and quercetin, were determined in a freeze-dried plant sample aliquot of 50 mg which was extracted with a methanol/water solution 80/20 (v/v) containing 10 mM NaF. The extract was analyzed by a Shimadzu Prominence HPLC system coupled with an AB Sciex 3200QTrap™ mass detector.

Total polyphenols were spectrophotometrically determined at λ=740 nm on the plant extract with the F-C method using chlorogenic acid as reference standard.

Antiradical activity was spectrophotometrically determined at λ = 517 nm in 1 ml of sample extract using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Results

Effects of plant exposure to Cr(VI) and Cd(II)

Dose response analysis. The effect of the treatment with different concentrations of Cr and Cd on survival and callus formation capacity of *Nicotiana langsdorffii* leaf explants, are reported in Figure 1A. Increasing concentrations of both metals in the LS0 culture medium induced a significant decrease of leaf explants survival, and the total inhibition of callus formation capacity. A clear (visual symptoms) effect of plant growth inhibition was observed after fifteen days of treatment with metals (Figure 1). Particularly, treatments with 30 ppm Cd and 50 ppm Cr induced the reduction of plant growth while higher concentrations caused the death of plants as evidenced by the appearance of large necrotic areas on the leaves surface. On the basis of these results, the concentrations of 30 ppm Cd and 50 ppm Cr, both doses close to LD_{50}, were chosen to induce metal stress on *N. langsdorffii* wild

type plants and T_1 *N. langsdorffii* plants transgenic for the GR receptor. The latter prior to the treatment with metals were screened for the presence and the expression of the *gr* transgene. PCR and RT-PCR experiments amplified respectively from genomic DNA and RNA a fragment of the expected size in all the examined samples (data not shown). Non transformed and transgenic *Nicotiana* plants were thus treated with the selected metal concentrations for fifteen days and then analysed for a series of parameters indicative of the plant response to abiotic stress.

Leaf biomass of wild type and transgenic plants Figure 2 shows dry weight (d.w.), fresh weight (f.w.) and their percentage ratio of leaf biomass of *Nicotiana langsdorffii* both wild type and transgenic, exposed or not to 30 ppm Cd or 50 ppm Cr. Transgenic control plants showed an average sample fresh weight 30% lower than wild type ones, whereas the corresponding dry weights were closer and the difference was not statistically significant (p=0.01). A lower water content was therefore found in transformed plants, since the percentage of dry over fresh weight in the latter was 8.0%, that is about two-fold the value of wild type.

Metal stress of wild type gave rise to a decrease of leaf weight compared to control that resulted much more marked for chromium. In fact, the decrease of f.w. and d.w. was 73% and 59% for chromium, and 27% and 24% for cadmium, the latter being not statistically significant at p=0.01. Surprisingly, in transgenic plants cadmium exposure induced a leaf f.w. increase of 76%, highlighting a plant growth improvement, and even more surprisingly chromium did not exert any significant leaf biomass variation.

Analysis and uptake of Cr and Cd. Figure 3 shows chromium and cadmium content of *Nicotiana langsdorffii* both wild type and transgenic, exposed or not to 30 ppm Cd or 50 ppm Cr. Results evidenced a Cr and Cd content in control samples always lower than the detection limit (0.002 mg/g) and a metal uptake of wild type significantly higher than transgenic ones (Cr +25% and Cd +38%).

Analysis of phytohormones. Figure 4 shows abscisic acid (S-ABA), 3-indoleacetic acid (IAA) and salicylic acid (SAL) concentration of *Nicotiana langsdorffii* both wild type and transgenic, exposed or not to 30 ppm Cd or 50 ppm Cr.

Transformation of *N. langsdorffii* with the *gr* gene leads to a very significant increase of S-ABA (+75%), IAA (+64%) and above all of SAL (+720%) concentration in respect to wild type.

Moreover, wild type and transgenic genotypes behaved in a very different manner when exposed to toxic metals. The most evident difference is a marked increase of phytohormone content, compared with controls, observed in *N. langsdorffii* wild type exposed to 50 ppm Cr (S-ABA + 283%, IAA + 200%, and - once again - above all SAL +1080% which increases more than one order of magnitude) and to 30 ppm Cd (S-ABA + 83%, IAA + 71% and surprisingly SAL unvaried), while transgenic plants in the same experimental conditions showed a S-ABA ad IAA content which were not statistically different (p=0.01) in respect to controls, and a consistent depression of SAL content (-55% for Cr and – 83% for Cd).

Analysis of polyphenols and shikimic acid

Figure 5 shows the concentration of total polyphenols (TPH), chlorogenic acid (CLA), DPPH antiradical activity (RSA_{sample}) and shikimic acid (SHI) of *Nicotiana langsdorffii* both wild type and transgenic, exposed or not to 30 ppm Cd or 50 ppm Cr. Chlorogenic acid was found to be by far the most abundant compound in all the analyzed wild type *Nicotiana langsdorffii* extracts (1.9-7.8 mg/g d.w.). Among other monomeric polyphenols, scopoletin and caffeic acid were also found, at much lower concentration range: 7-2 and 4-6 μg/g d.w., respectively. TPH concentrations were linearly correlated with RSA_{sample} measured in the extracts ($R^2 = 0.981$, $p < 0.01$), evidencing the role of these compounds in the protection of the plant against free radicals originated by stress factors. A similar linear relationship was found between RSA_{sample} values and chlorogenic acid content.

Analogously to phytohormones, transformation of *N. langsdorffii* with the *gr* gene leads to a very marked increase of TPH (+122%), CLA (+247%) and SRA (+164%) concentration in respect to wild type plants, whereas SHI is practically unvaried. Accordingly, chlorogenic acid accounted for 33% of F-C total polyphenols in transgenic controls whereas in wild type control represented only 19%.

Once again, wild type and transgenic genotypes behaved in a very different manner when exposed to toxic metals. The most evident difference is a marked increase of TPH, CLA, SRA and SHI content, compared with controls, observed in *N. langsdorffii* wild type exposed to 50 ppm Cr (TPH + 112%, CLA + 271%, SRA + 150%, SHI + 214%. These findings confirmed the role of chlorogenic acid in the resistance mechanism of this species against abiotic stress. Conversely, transgenic plants exposed to Cr showed, in respect to control, an unvaried level of TPH, CLA and RSA. This result was in agreement with the biomass data, since the mean fresh and dry foliar biomass of transgenic plants treated with chromium was statistically comparable to the corresponding control and did not highlight any stress symptom. A consistent increase was observed only for SHI (+ 200%).

Exposure of wild type to 30 ppm Cd showed a modest but statistically significant ($p=0.01$) 15% increase of total polyphenols, and evidenced a rather low influence of Cd stress on polyphenol and SHI synthesis. This conclusion was also supported by chlorogenic acid and RSAsample data that exhibited a lowering in samples treated with cadmium than in controls and chlorogenic acid accounted for very similar percentages (15-19%) of total polyphenols in the two theses.

Finally, exposure of transgenic type to 30 ppm Cd left SHI almost unvaried, but caused a statistically significant ($p=0.01$) suppression, in respect to control, of TPH – 35%, CLA – 34% and SRA – 40%.

Discussion

Leaf biomass data clearly evidenced the surprising behaviour of genetically modified plants that were much more resistant than wild type organisms to Cd and Cr exposure at a concentration level in the growing medium as high as 30 and 50 ppm, respectively, that are concentrations usually able to induce metabolic modifications as strong as to inhibit plant growth in several species [6,9,11,13]. In this regard, it should be noted that the enhanced resistance of transgenic organisms to high Cd and Cr concentrations is in agreement with their minor metal uptake compared to wild type plants (Figure 3).

The higher polyphenol concentrations determined in transgenic control organisms compared to wild type ones, mimicking the metabolic spectrum observed in plants reacting to other kinds of

stress, is coherent with the fact that the gene insertion is a stress factor itself for Nicotiana langsdorffii and with the role of this compound class in its protection against stress phenomena.

As far as S-ABA and IAA contents both the wild type and transgenic plants not treated with metals confirmed the data obtained in the earlier paper [25], the transgenic genotype showing higher values of both phyto-hormones. On the other hand both Cd and Cr treatments induced an increase in hormone concentrations only in wild type plants, almost doubling those of transgenic *N. langsdorffii* in the case of Chromium. No significant differences have been found however between transgenic and wild type plants as far as shikimic acid concentrations were concerned, a significant increase being particularly evident in the case of Chromium treatment. However total polyphenols, chlorogenic acid and antiradical activity values were significantly higher in control (not treated with metals) and Cd treated GR plants all reaching the same high levels in both genotypes after Chromium treatment.

The general picture that can be drawn from the data just described seems to suggest that GR plants show a constitutive activation of defense responses possibly leading to a lower absorption of heavy metals while wild type ones will only be reacting in the presence of toxic agents. Plants do normally contain steroids (i.e. brassino-steroids) whose signaling chain starts from the recognition by the extra-cellular domain of a trans-membrane steroid binding receptor protein (BRI1) synergistic with a co-receptor signal transducer (BAK) [34,35]. Within the same system, a key role seems to be played by another membrane–binding protein, MSBP1, known to recognize also animal steroids like progesterone. Moreover, animal steroids have been shown to be present in plants and to be involved in plant growth regulation [36]. For instance, animal steroid hormones have been shown to induce flowering in wheat and *Arabidopsis* hypocotyl elongation, in peas, etc. Therefore it may be suggested that plant brassinosteroids may have a certain level of affinity with the rat gluco-corticoid receptor protein thus putatively being liable to be connected to their signaling chain. Brassinosteroids have been shown to induce tolerance to a wide range of abiotic stresses [37-41] through the activation of anti-oxydative stress systems [42], and the enhanced synthesis of abscisic acid [43], ethylene, salycilic acid [44], polyamines, indole-3-acetic acid [45,46]. Response to stress in plants, moreover, is known to be activated by increased levels of auxins with feedback interactions with flavonoid synthetic patterns [47,48] and crosstalk with brassinosteroid transcription (see [49] for an extensive review).

Conclusion

All together the experimental results clearly show a marked difference between transgenic and wild type *Nicotiana langsdorffii* plants. The rat glucocorticoid receptor gene has been integrated into the *Nicotiana* genome with a constitutive CaMV promoter and therefore will be constitutively transcribed. This would mean that plant brassinosteroids can constitutively activate the synthesis of auxins and, in connection with them induce a constitutive high concentration of active response related molecules like polyphenols and salycilic acid, and lowering metal absorption levels. Under metal stress however, it may be suggested that hyper-activation of flavonoid synthesis and in general of the defense systems could negatively interfere with the already present high level of stress responsive molecules and therefore inhibit the process only in GR transformed plants. This behavior would in this case be similar to the negative regulation of brassino-steroid signaling in the presence of over expression of the Membrane Steroid-Binding Protein 1 (MSBP1) shown by Yang et al. [34] and Song et al. [50], in *Arabidopsis*.

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