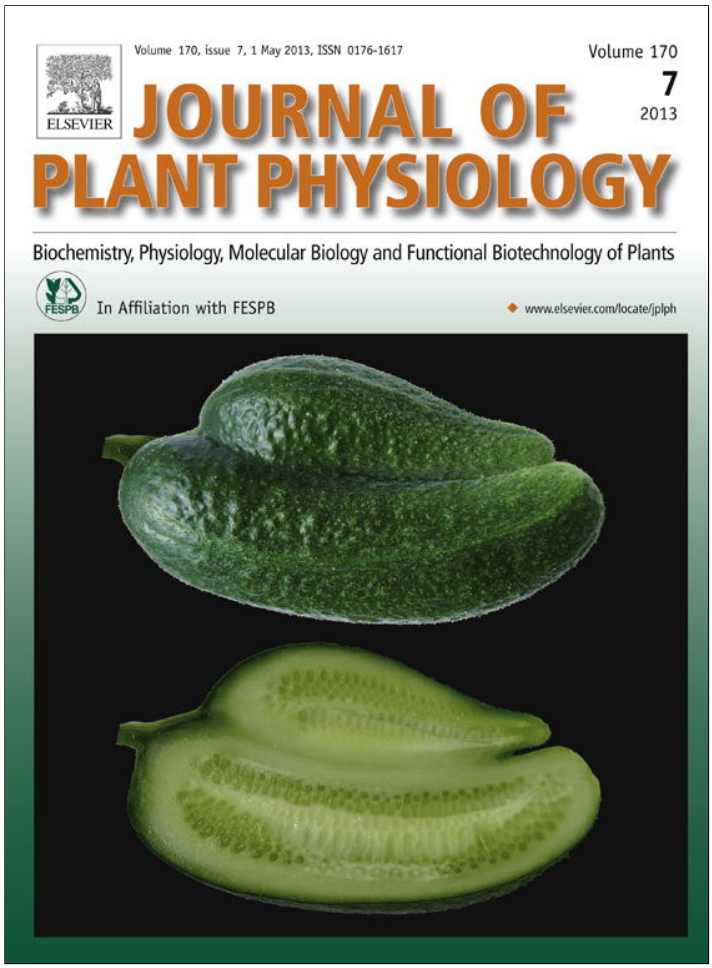

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Physiology

Response to metal stress of *Nicotiana langsdorffii* plants wild-type and transgenic for the rat glucocorticoid receptor gene

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ABSTRACT

Recently our findings have shown that the integration of the gene coding for the rat gluco-corticoid receptor (GR receptor) in *Nicotiana langsdorffii* plants induced morphophysiological effects in transgenic plants through the modification of their hormonal pattern. Phytohormones play a key role in plant responses to many different biotic and abiotic stresses since a modified hormonal profile up-regulates the activation of secondary metabolites involved in the response to stress. In this work transgenic GR plants and isogenic wild type genotypes were exposed to metal stress by treating them with 30 ppm cadmium(II) or 50 ppm chromium(VI). Hormonal patterns along with changes in key response related metabolites were then monitored and compared. Heavy metal up-take was found to be lower in the GR plants. The transgenic plants exhibited higher values of S-abscisic acid (S-ABA) and 3-indole acetic acid (IAA), salicylic acid and total polyphenols, chlorogenic acid and antiradical activity, compared to the untransformed wild type plants. Both Cd and Cr treatments led to an increase in hormone concentrations and secondary metabolites only in wild type plants. Analysis of the results suggests that the stress responses due to changes in the plant's hormonal system may derive from the interaction between the GR receptor and phytosteroids, which are known to play a key role in plant physiology and development.

Introduction

Plants have developed a complex system of responses to stress, the activation and dynamics of which depend not only on the duration and severity of the stress but also on the plant species, genotype and developmental stage. Response processes are specific for each different stress conditions as shown by the lack of any uniform transcriptome profile of plants subjected to different types of stress such as heat or cold, drought or salinity, strong light, mechanical and heavy metal stress (Cheong et al., 2002; Debnath et al., 2011; Kreps et al., 2002; Mantri et al., 2012; Marco et al., 2011; Rizhsky et al., 2004; Shen et al., 2012; Van de Mortel et al., 2008; Yang and Chu, 2011). Integrated responses to different biotic and abiotic stress conditions are regulated by the hormonal network of the plants, including auxins, cytokinins, abscisic acid (ABA), 3-indole acetic acid (IAA) and brassinosteroids (Bajguz and Hayat, 2009), which combined with many metabolites (*i.e.*, jasmonic acid and ethylene) and secondary messengers (*i.e.*, Ca^{2+}) play a pleiotropic regulatory key role. Thus, plant perception of environmental stress signals is initiated by recognition through specific receptors followed by signal transduction chains leading to the activation of the stress response network (Bray et al., 2000).

However, stress conditions can also be induced in plants by changes in the genetic, hormonal and physiological networks. 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 (Bettini et al., 2003, 2010) and in *rol*-transformed cells of various *Solanaceae*, *Araliaceae*, *Rubiaceae*, *Vitaceae* and *Rosaceae*. In fact, the modified hormonal profile up-regulates the activation of secondary metabolites involved in stress responses (Bulgakov, 2008). These results combined with other findings show that the components of the overall hormonal network are dynamically connected and therefore any change to any

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of them may lead to modifications in the network as in the initiation of responses to stresses. Among abiotic stresses, the following cause some of the most severe damages to plants: light, water-logging, drought, heat, cold, salinity, and the presence of heavy metals (Nies, 1999; Schützendübel and Polle, 2002). Of these, Cd is highly phytotoxic and can cause plant death (Wójcik and Tukiendore, 2005; Wahid et al., 2009). Low ppm Cd concentrations have an inhibitory effect on root and shoot growth in several plant species by affecting the functionality of membranes, related enzymatic activities (Fodor et al., 1995; Ouariti et al., 1997), and the photosynthesis rate (Lozano-Rodriguez et al., 1997; Sandalio et al., 2001; Somashekaraiah et al., 1992). These in turn alter the uptake and distribution of macro and micro-nutrients (Gussarson et al., 1996; Lozano-Rodriguez et al., 1997; Sandalio et al., 2001). Oxidative stress has also been related to Cd exposure (Guan et al., 2009).

The toxic effect related to chromium exposure is strongly dependent on the oxidation state and on the plant's ability to accumulate Cr (Zayed and Terry, 2003). In fact, Cr(VI) is by far more toxic than Cr(III) (Shanker et al., 2005), although Cr is poorly translocated to aerial parts and is mobilized and accumulated in tissues differently depending on its chemical form (James and Bartlett, 1983). Cr(III) does not show any measurable toxic effect even at several tens of ppm in the culture medium (Moral et al., 1995, 1996). The efficiency of Cr(III) adsorption by the radical apparatus and the mobility in the plant depend on the presence of complexing agents (James and Bartlett, 1983; Bluskov et al., 2005). Conversely, chromate ions are able to cross the plasmatic membrane and can cause dose-related damages to the plant although most of Cr(VI) is eventually reduced to the less toxic trivalent form. A Cr(VI) concentration higher than 1–10 ppm in the culture medium produces growth inhibition, a decrease in chlorophyll synthesis, a reduction in photosynthesis and chlorosis inhibition of mineral absorption in various plants (Shanker et al., 2005; Moral et al., 1995, 1996; Dube et al., 2003). In particular, 10 ppm in the culture medium of *citrullus* plants had a clearly negative effect on plant development (Dube et al., 2003), which was related to metabolic alterations due to a direct effect on enzymes or other metabolites or due to the generation of reactive oxygen species which may cause oxidative stress (Polle and Rennenberg, 1992).

In an earlier paper we reported the effects of the integration into *Nicotiana langsdorffii* and *Nicotiana glauca* plants of the gene coding for the rat glucocorticoid receptor (GR receptor) on phytohormone levels. GR receptor in animals acts as a steroid-dependent transcription factor, regulating the expression of several glucocorticoid responsive genes that control development, organ physiology, cell differentiation and homeostasis (Mangelsdorf et al., 1995). Our results showed that the integration of the *gr* transgene into the genome of the two *Nicotiana* species induced pleiotropic morphological and physiological changes deriving from the modification of their hormonal pattern (Giannarelli et al., 2010). Auxin content increased in the first species and decreased in the second species, thus drastically changing the cytokinin/auxin ratio in both cases. This is in line with the pre-existing different phytohormone balances of the two species (Bayer, 1982).

The aim of this paper was therefore to investigate whether the observed differences between the background hormonal patterns of *N. langsdorffii* wild type and transgenic for the GR receptor, influenced plant responses to Cr(VI) or Cd(II) exposure through changes in the structure and dynamics of the metabolic network. Hormonal patterns, along with the changes in key response-related metabolites, were thus monitored in both genotypes exposed and unexposed to 30 ppm Cd(II) or 50 ppm Cr (VI). The concentrations of phytohormones such as S-abscisic acid (S-ABA) and 3-indole acetic acid (IAA) along with salicylic acid, a known inducer of active defense responses, shikimic acid, a key molecule in the polyphenol synthetic pathway, and polyphenols themselves (known to be secondary metabolites with a key role in the response of plants to stress), were accurately measured. Finally, metal uptake was also evaluated both in exposed and unexposed wild type and transgenic plants.

Materials and methods

Plant material and nucleic acid extraction

Transgenic *Nicotiana langsdorffii-GR* plants have been described elsewhere (Giannarelli et al., 2010). Leaf disk transformation experiments were performed using *Agrobacterium tumefaciens* strain LBA4404 and the binary vector pTI18 bearing the rat glucocorticoid receptor (*gr*) gene (Irdani et al., 1998). 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. T_0 plants were thus kept in a greenhouse under natural lighting with a day length of 16 h and temperature ranging from 18 °C to 24 ± 1 °C. Transgenic plants were then allowed to self-pollinate. Harvested T_1 seeds were surface sterilized with commercial sodium hypochlorite for 20 min, washed three times with distilled water and placed into *Petri* dishes containing LS medium (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 LS medium for a month. Each plant was then screened for the presence and the expression of the *gr* gene and then multiplied by cutting internodes containing stem fragments for further analyses.

Genomic DNA was isolated from the young leaves of each T_1 plant and from *N. langsdorffii* not transformed by using the commercially available NucleoSpin® Plant II kit (Macherey-Nagel, M-Medical, Italy). Total RNA was isolated with a Plant RNA extraction kit (Macherey-Nagel, M-Medical, Italy) according to the manufacturer's instructions. The extracted nucleic acids were then respectively used for PCR and RT-PCR amplification of the *gr* gene as described in the literature (Giannarelli et al., 2010).

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 the survival and callus formation capacity of leaf tissues of wild type *N. langsdorffii* plants grown *in vitro*. The standard working solutions were prepared from potassium dichromate ($K_2Cr_2O_7$) and cadmium sulphate ($CdSO_4$) standard solutions (Merck Titrisol). Working solution were filter sterilised using 0.22 μm Millipore membrane filters and added to the LS medium in different concentrations. Thirty leaf disks with a diameter of 12 mm were placed into Petri dishes containing a 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) or cadmium (0, 10, 30, 50 ppm) and incubated in the growth chamber at 24 ± 1 °C.

The survival efficiency and callus formation capacity of explants, expressed respectively as the percentage of green and callus-forming explants against controls, were determined at 15-day intervals for one month. Experiments were carried out in triplicate. In addition, 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 Netherlands) with LS medium containing the metal at the selected concentration level. Leaf biomass was also determined before and after freeze-drying both the wild type and transgenic plants exposed and unexposed to 50 ppm Cr or 30 ppm Cd.

Sample analysis (see Supplementary material for details)

Total Cr and Cd concentrations were determined in a freeze-dried and homogenized 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 with an Inductively Coupled Plasma–Quadrupole Mass Spectrometer (ICP-QMS, Agilent 7500) equipped with a V-groove nebulizer and a cooled spray chamber.

Abscisic acid (ABA) and 3-indoleacetic acid (IAA) were determined in a freeze-dried and homogenized plant sample aliquot of 1.5 g by cold extraction (−28 °C) with a methanol/water/formic acid mixture (75:20:5 v/v). The final extract was analysed with a Perkin Elmer 200 liquid chromatograph coupled with an Analytical Biosystem Sciex mod API 4000 mass spectrometer, with a triple quadrupole and turbo spray ion source.

Shikimic acid (SHI) and salicylic acid (SA) 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 an Agilent series 4100 liquid chromatograph coupled with an Analytical Biosystem Sciex mod. API 4000 mass spectrometer, with a triple quadrupole and turbo spray ion source.

Selected monomeric polyphenols, namely 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 with a Shimadzu Prominence HPLC system coupled with an AB Sciex 3200QTrap™ mass detector.

Total polyphenols were spectrophotometrically determined at $\lambda = 740$ nm on the plant extract with the Folin–Ciocalteau (F-C) method using chlorogenic acid as a reference standard.

Antiradical activity (RSA) was spectrophotometrically determined at $\lambda = 517$ 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

Fig. 1 shows the effect of the treatment with different concentrations of Cr and Cd on the survival and callus formation capacity of *N. langsdorffii* leaf explants. Increasing concentrations of both metals in the LS culture medium significantly decreased leaf explant survival, and totally inhibited callus formation. A clear (visual symptoms) effect of plant growth inhibition was observed after 15 days of treatment with metals (Fig. 1). Treatments with 30 ppm Cd or 50 ppm Cr reduced plant growth, while higher concentrations caused the death of plants as evidenced by the appearance of large necrotic areas on leaf 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* plants, both wild type and transgenic for the GR receptor. The latter were screened for the presence and expression of the *gr* transgene prior to treatment with metals. PCR and RT-PCR experiments revealed a fragment of the expected size amplified from all the samples of transgenic plants examined (data not shown). Non transformed and transgenic *Nicotiana* plants were thus treated with the selected metal concentrations for 15 days and then analysed for a series of parameters indicative of plant response to abiotic stress.

Leaf biomass of wild type and transgenic plant

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

Metal stress in the wild type led to a decrease in leaf weight compared to the controls. This decrease was much stronger in the case of chromium exposure. In fact, the decrease in f.w. and d.w. was 73% and 59% for chromium, and 27% and 24% for cadmium, the difference between the latter not being statistically significant at $p = 0.01$. Transgenic plants were not significantly affected by Cr and Cd treatments as highlighted by both fresh and dry weight data.

Analysis and uptake of Cr and Cd

Fig. 3 shows the chromium and cadmium content of both wild type and transgenic *N. langsdorffii* exposed and unexposed to 30 ppm Cd or 50 ppm Cr. Results evidenced Cr and Cd content in the control samples which was always under the detection limit (0.002 mg/g). In addition, the metal uptake by wild-type plants was significantly higher than that in transgenic plants (Cr +25% and Cd +38%).

Analysis of phytohormones

Fig. 4 shows abscisic acid (S-ABA), 3-indoleacetic acid (IAA) and salicylic acid (SA) concentrations of both wild type and transgenic *N. langsdorffii*, exposed and unexposed to 30 ppm Cd or 50 ppm Cr. Transformation of *N. langsdorffii* with the *gr* gene led to a very significant increase in S-ABA (+75%), IAA (+64%) and especially of SA (+720%) concentrations in comparison with wild type plants. Moreover, wild type and transgenic genotypes behaved very differently when exposed to toxic metals. The most evident difference compared with controls, was a marked increase in phytohormone content in *N. langsdorffii* wild type exposed to 50 ppm Cr (S-ABA + 283%, IAA + 200%, and – again – especially SA + 1080% which corresponds to an increase of more than one order of magnitude) and to 30 ppm Cd (S-ABA + 83%, IAA + 71% and surprisingly SA was unvaried). Transgenic plants on the other hand, in the same experimental conditions showed S-ABA and IAA contents that were not statistically different ($p = 0.01$) compared to the controls, as well as a substantial decrease in SA content (−55% and −83% for Cr and Cd exposure, respectively).

Analysis of polyphenols, shikimic acid and RSA

Fig. 5 shows the concentration of total polyphenols (TPH), chlorogenic acid (CLA), DPPH antiradical activity (RSA_{sample}) and shikimic acid (SHI) of both wild type and transgenic *N. langsdorffii*, exposed and unexposed 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 *N. langsdorffii* samples (1.9–7.8 mg/g d.w.). Among the other monomer polyphenols, scopoletin and caffeic acid were found at a much lower concentration range: 7–20 and 4–6 μg/g d.w., respectively. A linear correlation was found between TPH concentration and the corresponding RSA_{sample} measured in the extracts ($R^2 = 0.981$, $p < 0.01$), highlighting the role of these compounds in the protection of the plant against free radicals caused

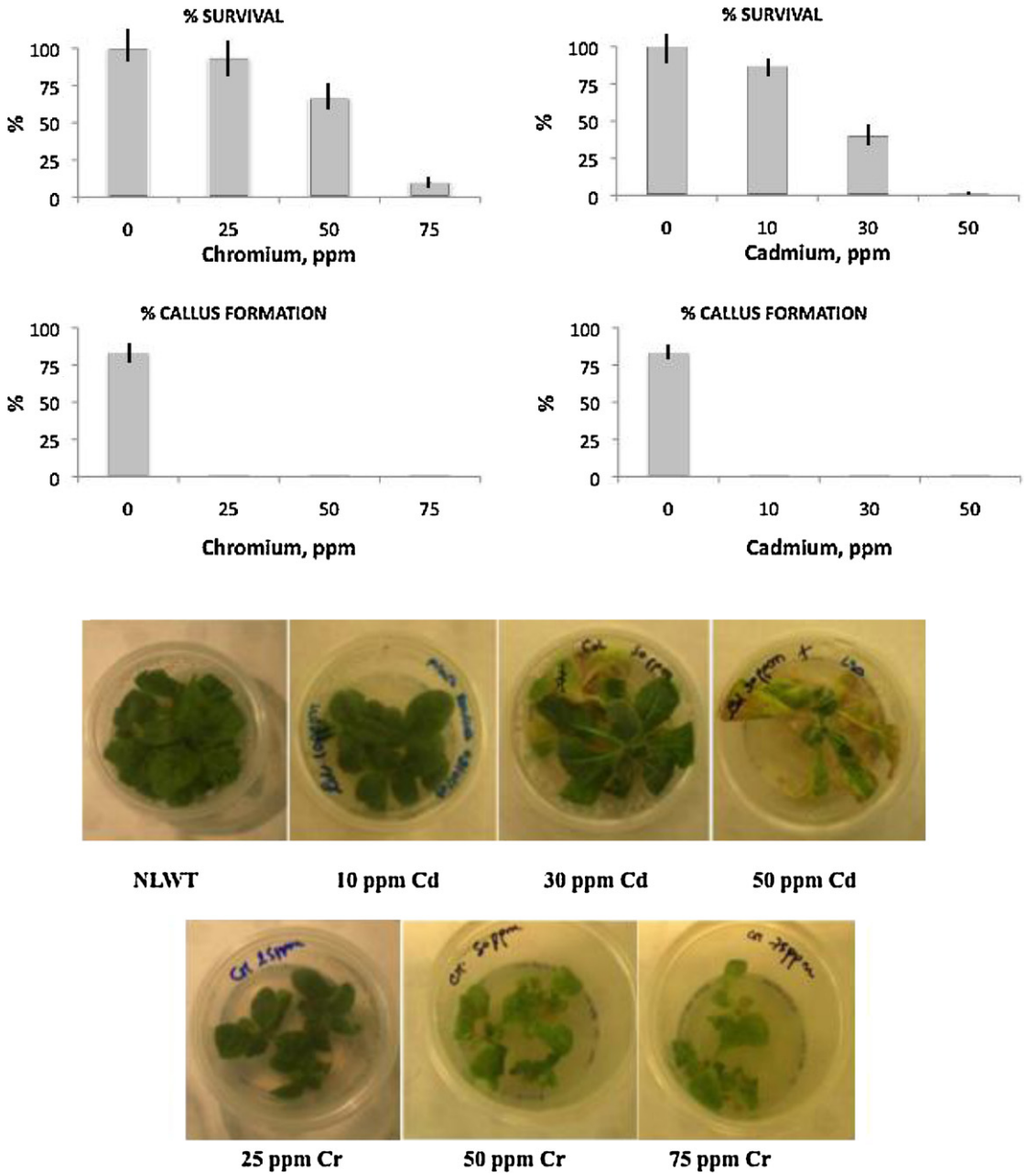

Fig. 1. Effect of different concentrations of Cr(VI) or Cd(II) on survival and callus formation of *N. langsdorffii* leaf explants determined after one month of culture and on *in vitro* growth of *Nicotiana* plants after 15 days of metal treatments. Error bars correspond to the standard deviation of three replicates (90 explants).

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 led to a very marked increase in TPH (+122%), CLA (+247%) and RSA (+164%) concentration compared to wild type plants, whereas SHI practically remained the same. Chlorogenic acid accounted for 33% of F–C total polyphenols in transgenic controls, whereas in wild type controls it represented only 19%.

Again, wild type and transgenic genotypes behaved very differently when exposed to toxic metals. The most evident difference compared with controls was the marked increase in TPH, CLA, RSA and SHI content in *N. langsdorffii* wild type exposed to 50 ppm Cr (TPH + 112%, CLA + 271%, RSA + 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 unchanged levels of TPH, CLA and RSA compared to the controls. This result was in agreement with the biomass data, since the mean fresh and dry leaf biomass of transgenic plants treated with chromium was statistically comparable to the corresponding control and did not highlight any stress symptom. A substantial 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 in total polyphenols, and highlighted a rather low influence of Cd stress on polyphenol and SHI synthesis. This conclusion was also supported by chlorogenic acid and RSA data which exhibited a reduction in the samples treated with cadmium compared to the controls, and chlorogenic acid accounted for very similar percentages (15–19%) of total polyphenols in both sample series.

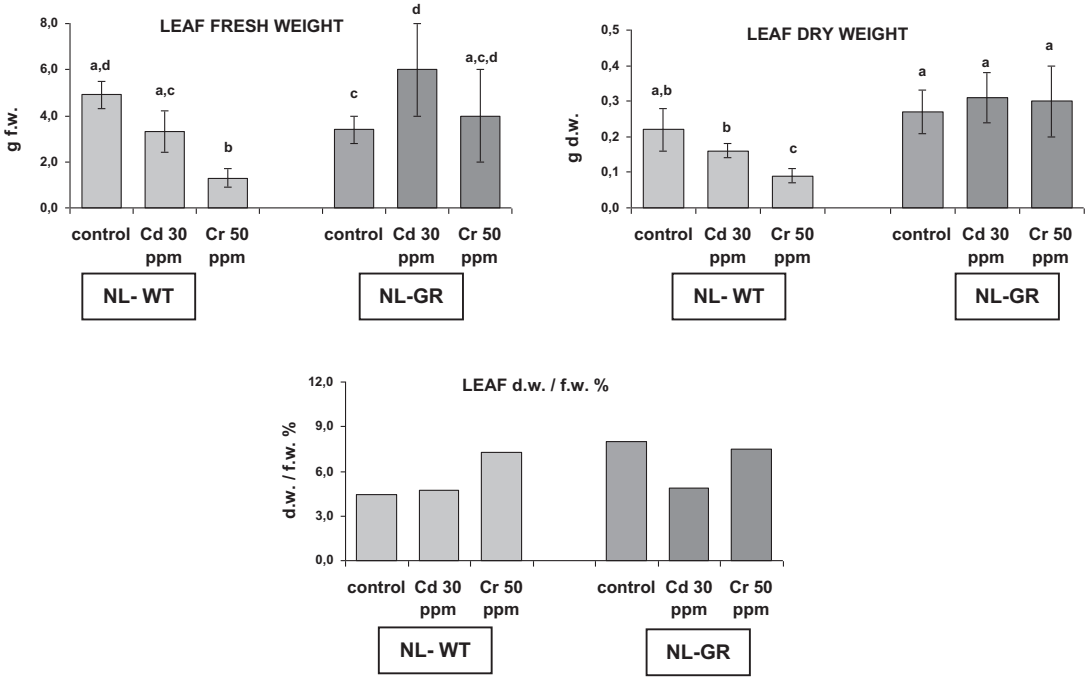

Fig. 2. Leaf fresh weight (f.w.), dry weight (d.w.) and d.w./f.w. percentage of wild type (NL-WT) and transformed (NL-GR) *N. langsdorffii* samples exposed and unexposed to 50 ppm Cr(VI) or 30 ppm Cd(II) in the culture medium. Error bars correspond to the standard deviation for $n = 5$. Values with the same letter were not statistically different at 1% significance level according to Tamhane test.

Finally, exposure of transgenic plants to 30 ppm Cd left SHI almost unvaried, but caused a statistically significant ($p = 0.01$) suppression of TPH–35%, CLA–34% and RSA–40% compared to the controls.

Discussion

A comparative evaluation of the phytohormone network of both wild type and transgenic *N. langsdorffii* for the rat glucocorticoid GR receptor, clearly shows strong differences in the responses to Cd or Cr exposure at a concentration level as high as 30 ppm and 50 ppm, respectively, in the growth medium. As far as phyto-hormones are concerned, S-ABA and IAA contents confirmed the data of our earlier paper (Giannarelli et al., 2010). In fact, the transgenic genotype showed higher values of both phytohormones than the wild type. In addition, leaf biomass data clearly showed a much higher level of resistance of genetically modified plants than the wild type to 30 ppm Cd or 50 ppm Cr exposure, both concentrations inhibiting *N. langsdorffii* plant growth. This result concurs with a lower metal uptake of transgenic plants.

Activation of stress responses was monitored in both genotypes through the evaluation of the synthesis of active defense metabolites such as polyphenols which are antioxidant molecules, deriving from the shikimic acid pathway. The metabolic spectrum observed for both the transgenic plants unexposed and exposed to Cr was very similar to that of wild type samples exposed to metal stress. In fact, they showed higher levels of total polyphenols, chlorogenic acid and antiradical activity which suggested a sort of permanent state of "alert" putatively due to the previously discussed constitutive changes in the hormone network (Giannarelli et al., 2010). This hypothesis is in line with the experimental evidence that hormone concentrations did indeed increase after treatment with chromium in wild type plants, almost doubling the levels found in transgenic *N. langsdorffii*. At variance with these data, no significant difference in shikimic acid concentrations was found between transgenic and wild type plants, a significant increase of which was found in plants of both genotypes after Cr exposure. The state of "alert" of *N. langsdorffii* genetically modified with the rat glucocorticoid receptor gene could be due to the different states of the phytohormone network in transgenic and wild type plants. It is

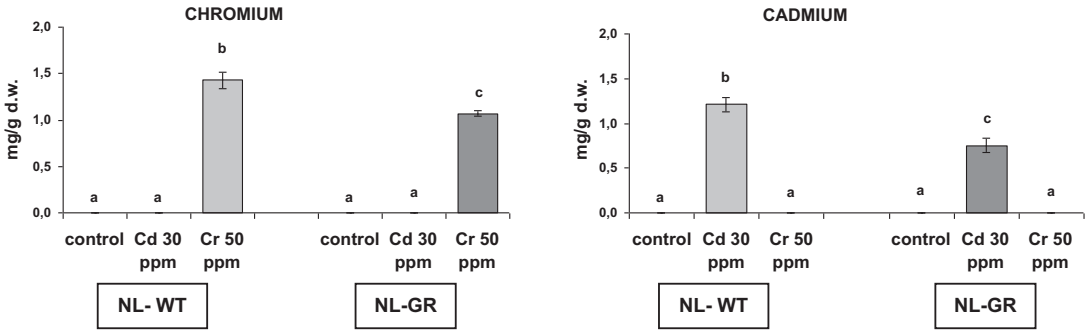

Fig. 3. Chromium and cadmium concentration in wild type (NL-WT) and transformed (NL-GR) *N. langsdorffii* samples exposed and unexposed to 50 ppm Cr(VI) or 30 ppm Cd(II) in the culture medium. Error bars correspond to the standard deviation for $n = 5$. Values with the same letter were not statistically different at 1% significance level according to Tamhane test.

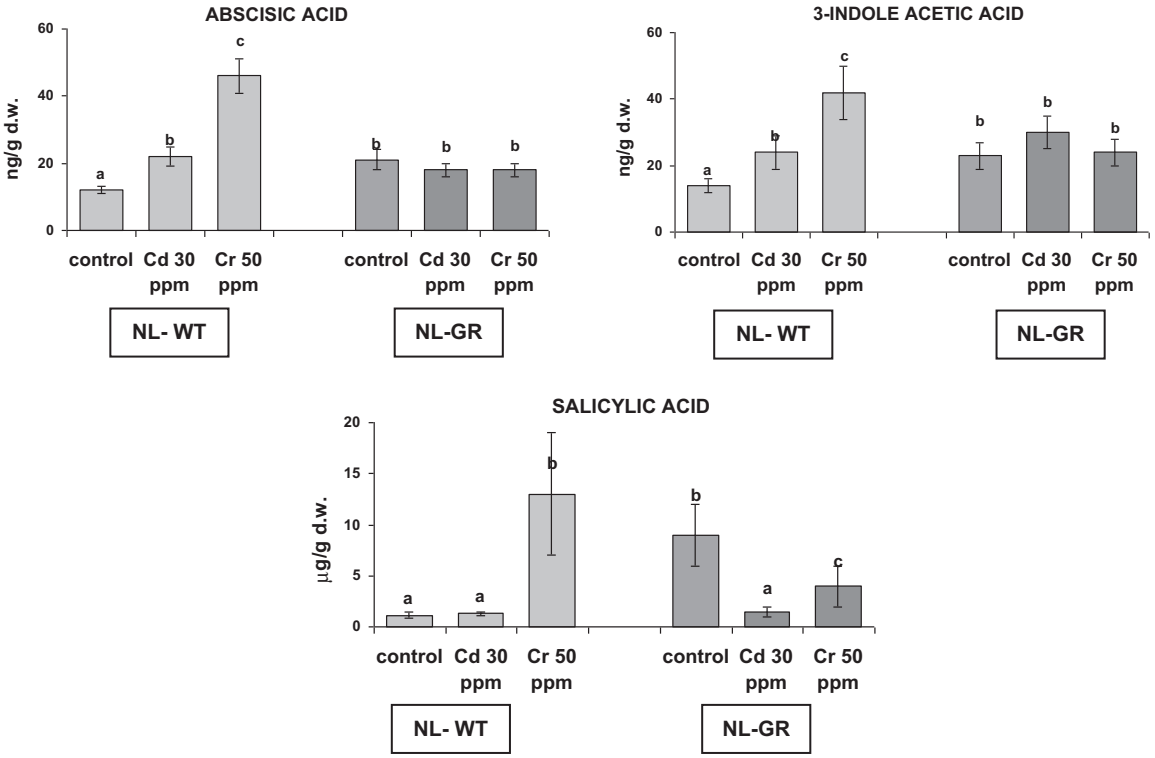

Fig. 4. Abscisic acid (S-ABA), 3-indoleacetic acid (IAA) and salicylic acid (SAL) concentration in wild type (NL-WT) and transformed (NL-GR) *N. langsdorffii* samples exposed and unexposed to 50 ppm Cr(VI) or 30 ppm Cd(II) in the culture medium. Error bars correspond to the standard deviation for $n = 5$. Values with the same letter were not statistically different at 1% significance level according to Tamhane test.

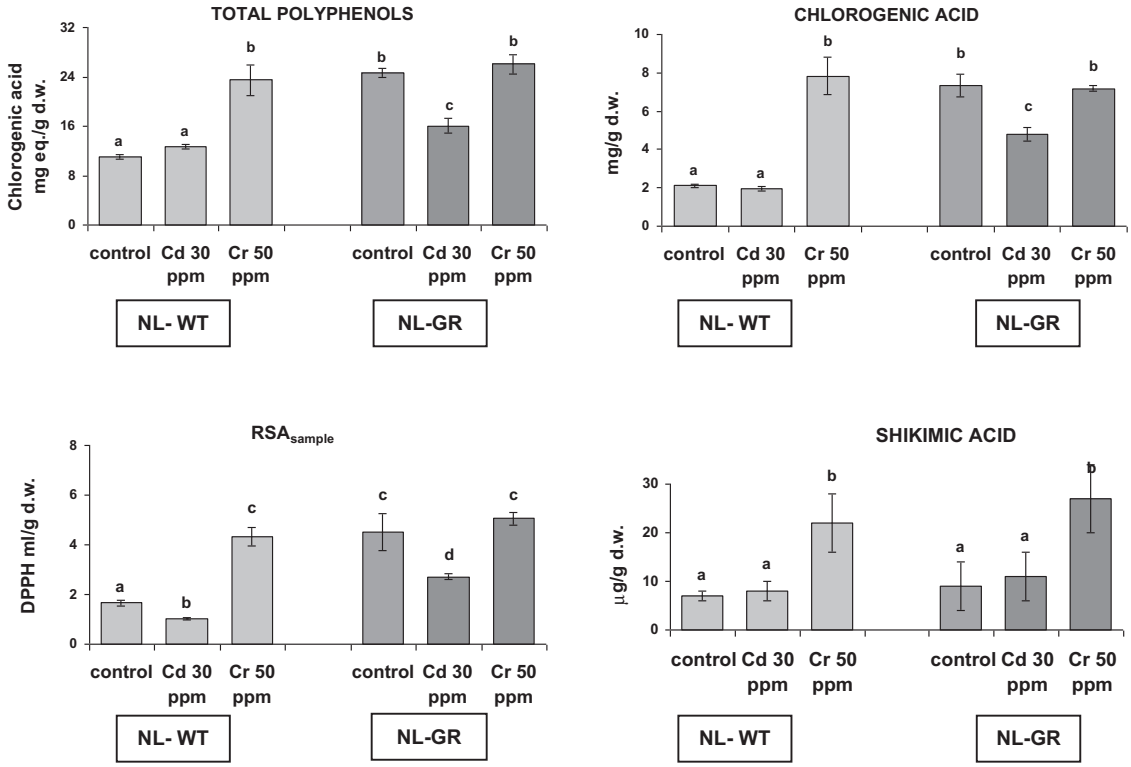

Fig. 5. Polyphenols, chlorogenic acid, RSA_{sample} and shikimic acid concentration in wild type (NL-WT) and transformed (NL-GR) *N. langsdorffii* samples exposed and unexposed to 50 ppm Cr(VI) or 30 ppm Cd(II) in the culture medium. Error bars correspond to the standard deviation for $n = 5$. Values with the same letter were not statistically different at 1% significance level according to Tamhane test.

worth recalling here that: (i) plants normally contain steroids (*i.e.* brassinosteroids) whose signaling chain starts from the recognition of a trans-membrane steroid binding receptor protein (BRI1) by the extra-cellular domain, which is synergistic with a co-receptor signal transducer (BAK) (Yang et al., 2005, 2008); (ii) within the same system, a key role seems to be played by another membrane steroid-binding protein, MSBP1, known to also recognize animal steroids such as progesterone. Animal steroids have also been shown to be present in plants and to be involved in plant growth regulation (Iino et al., 2007), inducing for instance flowering in wheat and *Arabidopsis* hypocotyl elongation. Therefore, it is possible that plant brassinosteroids have sufficient affinity with the rat gluco-corticoid receptor protein to be connected to their signaling chain through the recognition of molecules expressed by the constitutive CaMV promoter inserted in the transformation construct. Brassinosteroids have been shown to induce tolerance to a wide range of abiotic stresses (Kagale et al., 2007; Bajguz, 2011; Sittayat et al., 2007; Arora et al., 2010a; Koh et al., 2007) through the activation of anti-oxidative stress systems (Arora et al., 2010b), as well as the enhanced synthesis of abscisic acid (Yuan et al., 2010), ethylene, salicylic acid (Divi et al., 2010), polyamines and indole-3-acetic acid (Choudhary et al., 2010, 2011). Responses to stress in plants, moreover, are known to be activated by increased levels of auxins, which in turn determines feedback interaction with flavonoid synthetic patterns (Peer and Murphy, 2007; Lewis et al., 2011) and crosstalk with brassinosteroid transcription process (see Depuydt and Hardtke (2011) for an extensive review).

Under metal stress however, the hyper-activation of flavonoid synthesis and of the defense systems in general could negatively interfere with the already present high levels of stress responsive molecules and therefore inhibit the process only in GR transformed plants. This behavior is similar to the negative regulation of brassinosteroid signaling in the presence of over expression of the Membrane Steroid-Binding Protein 1 (MSBP1) shown by Yang et al. (2005) and Song et al. (2009) in *Arabidopsis*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2012.12.009.

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