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Research article

Simultaneous determination of shikimic acid, salicylic acid and jasmonic acid in wild and transgenic *Nicotiana langsdorffii* plants exposed to abiotic stresses

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ABSTRACT

The presence and relative concentration of phytohormones may be regarded as a good indicator of an organism's physiological state. The integration of the *rolC* gene from *Agrobacterium rhizogenes* and of the rat glucocorticoid receptor (*gr*) in *Nicotiana langsdorffii* Weinmann plants has shown to determine various physiological and metabolic effects. The analysis of wild and transgenic *N. langsdorffii* plants, exposed to different abiotic stresses (high temperature, water deficit, and high chromium concentrations) was conducted, in order to investigate the metabolic effects of the inserted genes in response to the applied stresses. The development of a new analytical procedure was necessary, in order to assure the simultaneous determination of analytes and to obtain an adequately low limit of quantification. For the first time, a sensitive HPLC-HRMS quantitative method for the simultaneous determination of salicylic acid, jasmonic acid and shikimic acid was developed and validated. The method was applied to 80 plant samples, permitting the evaluation of plant stress responses and highlighting some metabolic mechanisms. Salicylic, jasmonic and shikimic acids proved to be suitable for the comprehension of plant stress responses. Chemical and heat stresses showed to induce the highest changes in plant hormonal status, differently affecting plant response. The potential of each genetic modification toward the applied stresses was marked and particularly the resistance of the *gr* modified plants was evidenced. This work provides new information in the study of *N. langsdorffii* and transgenic organisms, which could be useful for the further application of these transgenes.

1. Introduction

When in adverse or limiting conditions, plants activate a complex system of responses in order to alleviate cellular damage and to survive (Fuoco et al., 2013). Water deficiency, high temperatures and pollution represent the main stress factors for plants in relation to the expected climate changes. Heat stress conditions affect the cell membranes and the enzyme functionality while modifying the transpiration rate (Lipiec et al., 2013); water deficiency determines the inhibition of photosynthesis, the enhancement of respiration and the lack of mineral nutrients (Yordanov et al., 2000). Heavy metals, due to their widespread distribution and their persistency, represent one of the main issues for agriculture and land use. Heavy metals such as cadmium and chromium (Cr) induce enzyme inhibition, cellular oxidation and the alteration of metabolism (Obata and Fernie, 2012). Cr(VI) is the most toxic oxidation state of chromium, whose uptake was shown to influence the plant's growth, the production of many essential metabolites and enzymatic activity (Singh et al., 2015).

The use of genetic engineering to produce transformed stress-resistant organisms is increasingly gaining interest. Among the genetic modifications studied, the integration of the gene codifying for the rat glucocorticoid receptor (*gr*), which regulates genes controlling the development, metabolism and immune response, appears to be promising, inducing higher resistance against

Abbreviations: ABA, Abscisic acid; ACN, acetonitrile; Cr, chromium; *gr*, glucocorticoid receptor; HS, heat stress; IS, internal standard; FR, instrumental response factor; JA, jasmonic acid; LOD, limit of detection; LOQ, limit of quantification; ME, matrix effect; MeOH, methanol; PTFE, polytetrafluoroethylene; PEG 6000, polyethylene glycol 6000; RSA, radical scavenging activity; ROS, reactive oxygen species; SA, salicylic acid; $SA^{13}C_6$, salicylic acid phenyl$^{13}C_6$; SHA, shikimic acid; WS, water stress; WT, wild type.

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nematode infections and chemical stress in *Nicotiana* plants (Del Bubba et al., 2013). The *rolC* gene is a plant oncogene carried on plasmids of the plant pathogen *Agrobacterium rhizogenes*; after infection, the gene can be transferred to the plant genome causing hairy root disease and tumor formation. Multiple biochemical and physiological alterations have been observed in *rolC* transformed plants, including stimulation of alkaloid, anthraquinone and cytokinin production (Bulgakov et al., 2008; Kiselev et al., 2006). Enhancement of plant response to abiotic and biotic stresses has even been related with *rolC* gene insertion (Del Bubba et al., 2013; Intrieri and Buiatti, 2001). The *Nicotiana* genus (family of *Solanaceae*) includes small, well-characterized plants, traditionally used as biological models for genetic and physiological studies; the genetic *rolC* and *gr* modifications of *Nicotiana langsdorffii* plants were previously investigated, yielding interesting results for the production of plants resistant to different stresses (Del Bubba et al., 2013; Fuoco et al., 2013; Ranaldo et al., 2015). The biological state of plants can be monitored through different parameters such as their morphology, anatomy, physiology and biochemistry. Since the response to environmental stresses is controlled by the hormonal network, the presence and relative concentration of hormones may be regarded as a good indicator of an organism's physiological condition. Among the complex hormonal signaling system of plants, the following molecules have been recognized as central components of the adaptation response.

Salicylic acid (SA) and jasmonic acid (JA) are hormones involved in plant growth and development; recent studies demonstrated that they are implicated as signaling compounds in response to both biotic and abiotic stresses (Clarke et al., 2009; Maksymiec, 2007; Maksymiec et al., 2005; Metwally et al., 2003; Pál et al., 2005); many studies showed that the relative concentrations of JA and SA are affected during drought, chemical and temperature stresses (Clarke et al., 2009; De Ollas et al., 2013; He et al., 2014; Maksymiec et al., 2005; Pál et al., 2005; Wang et al., 2010). Shikimic acid (SHA) is an important intermediate in plant metabolism and a key molecule in the biosynthesis of numerous secondary metabolites. The SHA pathway represents the central point for the production of many compounds involved in the principal functions of plant life, including defense, such as flavonoids, lignins, indole derivatives and many aromatic alkaloids. The SHA pathway leads also to the production of SA, through the first step of the phenylpropanoid pathway or directly from isochorismate. The targeted determination of these three compounds could be very useful in the investigation of the effects of *rolC* and *gr* insertion in *N. langsdorffii* and in the better comprehension of plant response towards abiotic stresses. The aim of this paper is to investigate the effects of Cr(VI) exposure, water deficiency and high temperature on wild and transgenic *N. langsdorffii* plants, through the analysis of selected metabolites, in order to highlight the influence of the inserted transgenes (*rolC* and *gr* genes) on plant stress responses. The morphological and physiological effects of *rolC* and *gr* insertion in *N. langsdorffii* plants, exposed to heat, water and chemical stresses, have been the subject of other studies (Bogani et al., 2015; Ancillotti et al., 2015).

Taking advantage from the use of HPLC-HRMS technology, the quantitative determination of SA, SHA and JA was performed. The development of a new analytical procedure was necessary, in order to assure the simultaneous determination of analytes, due to the limited available plant material, and to obtain a limit of quantification adequately low to fulfill the analyte concentrations. The use of a high-resolution detector permitted the accurate measurement of metabolite masses and the discrimination between the analytes and potential interfering compounds; therefore the sample treatment procedure was fast, not requiring the purification step, which is generally essential for biological matrices analyses.

To our knowledge, no method for the simultaneous determination of these three compounds has been reported yet. The comparative evaluation of phytohormonal changes, induced by different abiotic stress factors, in wild type and in *gr* and *rolC* plants, allowed us to study the different metabolic mechanisms involved in stress response, in order to identify the organisms more promisingly resistant to the applied stresses.

2. Material and methods

2.1. Chemicals

SHA, JA, SA, salicylic acid phenyl$^{13}C_6$ (SA$^{13}C_6$) and acetic acid HPLC grade were purchased from Sigma Aldrich® (Buchs, Switzerland). HPLC/MS-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Romil LDT (Cambridge, U.K.). Hydrochloric acid (HCl) 37% ACS was purchased from Carlo Erba Reagents (Milano, Italy). Ultrapure water (18.2 MΩ cm, 0.01 TOC) was produced using a Purelab Ultra System (Elga, High Wycombe, U.K.).

2.2. Stock and working solutions

Stock standard solutions (10 μg/μL) of SHA, JA, SA and SA$^{13}C_6$ were prepared in ACN. Working standard solutions were prepared by diluting the stock solutions to obtain concentrations of 0.97 ng/μL for SA$^{13}C_6$ and 10 ng/μL for SA, SHA and JA.

2.3. Plant material

Plants of *N. langsdorffii Weinmann* were cultivated *in vitro* by Dr. Patrizia Bogani in the Laboratory of Plant Genetics, Department of Evolutionary Biology of the University of Florence. Wild type plants (WT) were genetically modified by inserting two kinds of genes: the gene codifying for the rat glucocorticoid receptor (*gr* plants) and the *rolC* gene from *A. rhizogenes* (*rolC* plants). The procedure for obtaining these genetic modifications is well described in previous studies (Del Bubba et al., 2013; Fuoco et al., 2013). Genetic identical plants were obtained by withdrawing portions of stems containing the internodes. Each plant was screened for the presence and the expression of *gr* and *rolC* genes as previously described (Fuoco et al., 2013). Plants were grown *in vitro* until reaching 30 days, as earlier explained (Del Bubba et al., 2013; Fuoco et al., 2013; Giannarelli et al., 2010). Prior to analysis, plants were fast cleaned with distilled water to remove the LS medium residues and frozen in liquid nitrogen; the whole plants were then freeze-dried in an Edward machine and, after complete water evaporation, maintained at environmental temperature.

2.4. Stress inductions

The plants analyzed in this study were subjected to heat stress (HS) by means of the heat shock method, through exposure at 50 °C for 2 h inside a thermostatic chamber; the water stress (WS) was induced by subculturing plants for 15 days on 50 mL of LS medium conditioned with 50 mL of a 20% polyethylene glycol 6000 (PEG 6000) solution, in accordance with literature (Khalid et al., 2010). These conditions were selected after test studies, as previously explained (Ancillotti et al., 2015; Scalabrin et al., 2015). The heavy metal stress by chromium (Cr) was induced by growing plants for 15 days using an LS medium containing $K_2Cr_2O_7$ (50 mg/kg of hexavalent chromium), as described by Del Bubba et al., 2013 and on the basis of preliminary stress responses experiments (Fuoco et al., 2013).

2.5. Sample treatment protocol

Globally, 80 samples of *N. langsdorffii* (*gr* = 10, wt = 10, *rolC* = 10, *gr*-WS = 6, wt-WS = 5, *rolc*-WS = 4, wt-Cr = 10, *gr*-Cr = 11, *rolc*-Cr = 7, WT-HS = 3, *rolc*-HS = 2, *gr*-HS = 2) were analyzed; the number of samples for each genotype and stress depended on the availability of plant material (supplied by the University of Florence) and on the weight of plants. With respect to HS plants, due to the limited number (3 organisms for each genotype) and weight of these plants, all the available plant material was mixed, obtaining an homogenized pool which was analyzed in three (wt-HS) or two (*rolc*-HS, *gr*-HS) independent technical replicates. For phytohormone analysis, samples were ground and homogenized by using a ball mill equipped with two polytetrafluoroethylene (PTFE) vessels and grinder balls; samples were ground for 5 min with a vibration frequency of 20 Hz to achieve a final fineness of ≈ 5 μm. Briefly, 0.1 g of homogenized plant material was weighted and the internal standard (IS) $SA^{13}C_6$ was added. The samples were extracted three times with 1.5 mL of fresh MeOH acidified with HCl (99.9/0.1, v/v) by centrifugation for 20 min at 20,000 rcf (relative centrifugal force) at 25 °C; the supernatants were collected, unified and evaporated in a thermostated bath at 30 °C, under a gentle stream of nitrogen. Once a 0.5 mL was reached, 1 mL of water was added and the extracts were filtrated with a syringe PTFE filter (Ø 25 mm, 0.45 μm). Two fractions were prepared for LC-MS analysis by diluting the extracts 1 to 20 times for SHA and SA determination and 1 to 5 times for JA. Along with each set of samples, one blank sample (for a total amount of eight blanks) was analyzed; the blanks were subjected to all processing steps, in order to verify the eventual contribution to analyte concentrations deriving from sample handling; moreover, blanks were used to calculate the limits of detection and quantification (LOD and LOQ) of the analytical procedure. SA and JA were quantified by means of the IS method while SHA was determined by means of an external calibration curve (see Appendix A, paragraph S2, Table S1). For this reason, for each sample batch, a calibration curve of SHA was also analyzed, in order to effectuate a correct quantification and to minimize instrumental signal variations. All samples were analyzed on the same day of their extraction, to prevent compound degradation.

2.6. Instrumental method

The instrumental analyses were carried out on a UHPLC Dionex Ultimate3000 LC system coupled to an HESI-LTQ Orbitrap (Thermo Fisher Scientific, Waltham, USA). The analysis was conducted with the ESI ion source operating in negative polarity, in full scan mode at the resolving power of 30,000 full width at half maximum. The final instrumental selected parameters are reported in Appendix A, paragraph S1, Table S2. The quantification of SA and JA was performed by means of an instrumental response factor (FR) to reduce possible instrumental signal variations. The main analytical parameters for each compound are reported in Table 1. The chromatographic separation was performed on a C_{18} phase 4 μm Synergy Hydro-RP 80 Å, 50 × 2 mm (Phenomenex®, Torrance, California, USA) eluted with acetic acid 0.1% (Eluent A) and MeOH (Eluent B) at a flow rate of 200 μL/min. The column oven temperature was set at 30 °C. The selected chromatographic run was composed of an initial 2 min isocratic step (100% eluent A) for the elution of SHA, followed by a 5 min gradient phase (20%/min) until reaching 100% of eluent B. The elution of SA, $SA^{13}C_6$ and JA was obtained with a following isocratic phase (100% eluent B) of 3 min (Fig. 1); the final step, for column conditioning, takes 7 min at 100% eluent A.

2.7. Method validation (see Appendix A paragraph S2, Table S1)

The method was validated by verifying accuracy, precision, analyte recovery and instrumental linearity. The matrix effect (ME) was also estimated, in order to assess the most appropriate quantification method.

2.8. Data processing and statistics

Chromatograms were integrated with LCquant 2.6.1 software (Thermo Fisher Scientific, Waltham, USA). The extracted ion chromatograms were generated by considering a small mass range (±5 ppm), centered on the exact *m/z* of each analyte. Data elaborations were performed by Excel add-in Multibase 2015 package (Numerical Dynamics, Japan). Statistical differences between controls and treated samples were analyzed using Student's t-test by means of Statistica 8.0 (StatSoft, Inc., 2007). Differences were considered significant at a probability level of $p < 0.05$.

3. Results and discussion

3.1. Effect of rolC and gr insertion

The *gr*-modified plants were obtained by inserting a rat gene encoding the glucocorticoid receptor, containing the constitutive CaMV promoter; it is possible that plant phyto-steroids, and particularly brassinosteroids, which showed to induce tolerance towards oxidative, drought and heavy metal stresses (Bartwal et al., 2013), have sufficient affinity with the rat glucocorticoid receptor protein and, therefore, can be activated by this signaling chain in *gr* transgenic plants. The *gr* insertion in *N. langsdorffii* has shown to determine the reduction of plant size, the decrease of electrolyte leakage and the increase of water content (Bogani et al., 2015). The integration of the *rolC* gene in plants reduced plant size, electrolyte leakage and root and shoot dry biomass (Ancillotti et al., 2015), while increased phytoalexin production and the expression of pathogenesis-related proteins (Bulgakov et al., 2008); *rolC* insertion, moreover, has been related to an increased activity of cytokinins and to the induction of many secondary metabolite pathways (Kiselev et al., 2006).

In this study, the insertion of *rolC* gene affected the content of JA, which was reduced in this genotype in comparison to *gr* and wt plants. This result could be related to the ability of cytokinins to suppress the JA levels, in agreement with the observations of Sano et al., 1996. As expected, JA showed lower concentrations (1×10^1–1.8×10^2 ng/g d.w.) than SA and SHA (3×10^3 to 1.7×10^4 ng/g d.w. and 2.5×10^3 to 5.5×10^3 ng/g d.w.

Table 1
Accurate masses, ionization modus, internal standard used, retention time and maximum mass deviation of the analytes.

Compounds	Ionization modus	Retention time (min)	Accurate mass (*m/z*)	Internal standard	Mass deviations (ppm)
Shikimic acid	negative	1.05	173.0455	Not used	−2.88
Salicylic acid	negative	7.16	137.0244	$SA^{13}C_6$	−1.45
Jasmonic acid	negative	7.83	209.1183	$SA^{13}C_6$	−2.86

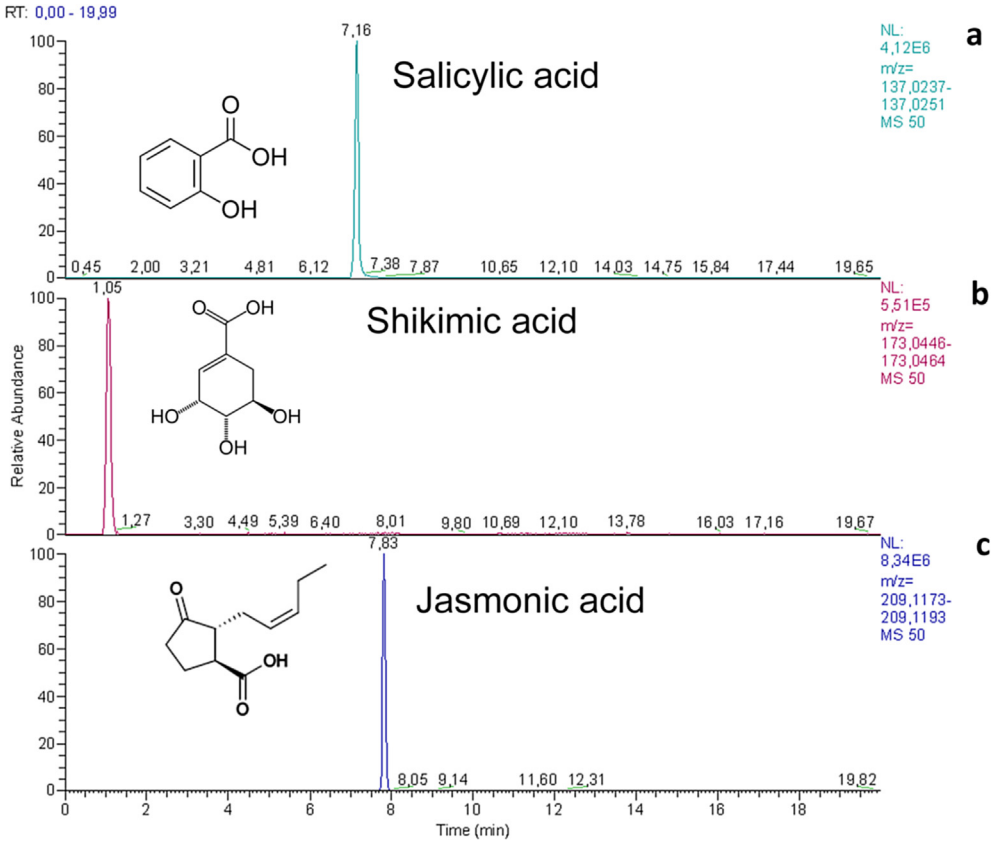

Fig. 1. Chromatographic separation of SA (a), SHA (b) and JA (c) in matrix enriched with standard solutions of analytes, at the concentration level of 50 pg/μL.

respectively). JA had the highest internal set variability, with relative standard deviations (RSD %) ranging from 34 in *rolC* plants to 66 in *gr* plants (Appendix A, Table S3). SA (RSD% = 32–34) and SHA (RSD% = 16–23) showed no statistically significant differences among the genotypes. The results are in agreement with previous studies (Schmelz et al., 2003; Fuoco et al., 2013). Interestingly, the concentration of SHA was similar in wt, *gr* and *rolC* plants, despite other studies suggested an increase of phenolic compound concentrations in transgenic plants (Del Bubba et al., 2013; Ancillotti et al., 2015); this finding indicates a different regulation of phenolics' biosynthesis in *gr* and *rolC* genotypes, probably through a modification of the phytohormonal balance in these organisms (Giannarelli et al., 2010).

3.2. Heat stress (HS)

The highest changes in analyte concentrations between controls and stressed samples were observed in plants exposed to HS. JA levels were increased 2.1 and 6.8 fold in wt-HS and *rolC*-HS plants respectively, while *gr*-HS plants showed unchanged concentrations of this compound.

The induction of JA has already been related with HS exposure, possibly caused by the disruption of cell membranes. Rizhsky et al., 2002, observed high lipoxygenase expression in *Nicotiana tabacum* during heat and drought stress, indicating the enhancement of JA biosynthesis. Endogenous JA biosynthesis and signaling, moreover, showed to be essential for thermotolerance in *Arabidopsis thaliana* (Clarke et al., 2009).

Interestingly, JA content in *gr* plants was not affected by HS exposure, suggesting a possible influence of this genetic modification on plant response toward heat stress. The unchanged concentrations of JA between *gr* and *gr*-HS plants could indicate a lighter damage of the photosynthetic system and of the membrane structures in this genotype, as confirmed by the limited changes in the lipidic profile of *gr* plants in comparison to *rolC* and wt genotypes (Scalabrin et al., 2015). This resistance could be due to the higher level of abscisic acid (ABA) in the *gr* genotype (Giannarelli et al., 2010), which might determine an earlier and more effective activation of plant defenses against this kind of stress (Kazan, 2015) in comparison to wt and *rolC*.

The phytohormone SA is known to take part in the defense against temperature stress, which primarily compromises the photosynthesis process; SA particularly promotes the activity of the Rubisco enzyme and increases the efficiency of the photosynthetic system during heat stress exposure (Wang et al., 2010). However, several studies reported an initial substantial increase of endogenous SA, in the first 30 min of the HS application, followed by a drastic decrease, indicating that the accumulation of SA represents an early signal in heat response (Pan et al., 2006). Nevertheless, the induction of SA has been explained as an indirect effect of heat stress exposure, as a consequence of ABA increase (Kurepin et al., 2013); moreover Clarke et al., 2009 highlighted the synergic/antagonistic interaction of SA and JA during heat stress.

In our study, 2 h exposure to 50 °C determined unchanged SA levels in *rolC*-HS and *gr*-HS plants, consistently with literature, while a significant decrease was observed in wt-HS plants (Fig. 2 a,b,c). SA decreased levels in wt plants could indicate a higher thermosensitivity of this genotype, in comparison to *gr* and *rolC*, whose biochemical status showed to be less affected by heat stress (Scalabrin et al., 2015). However, the mechanisms of regulation of SA levels and the interactions with JA, ABA and other phytohormones are not fully understood yet.

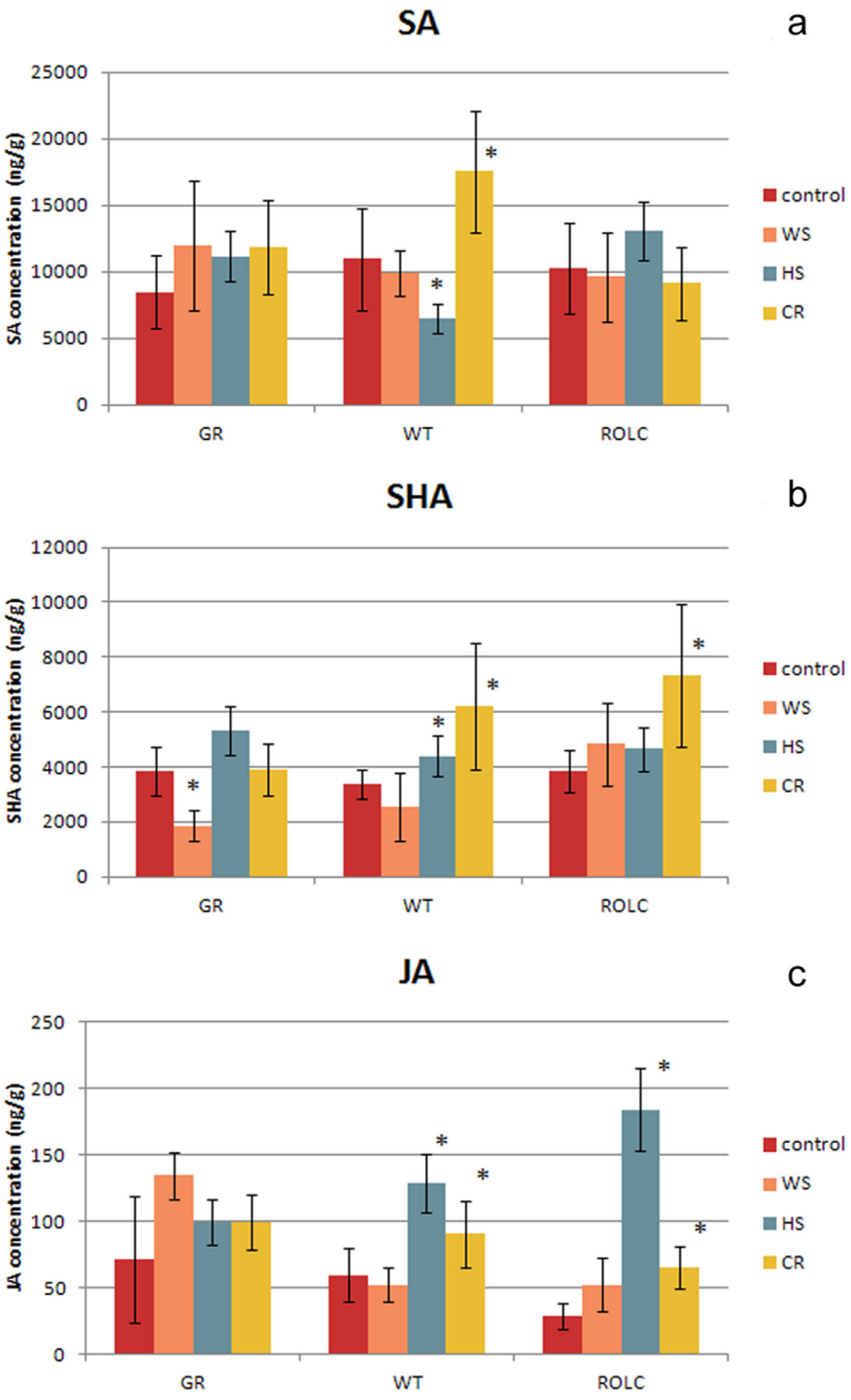

Fig. 2. a,b,c Mean phytohormone concentrations in controls, WS, HS and CR samples in wild, *gr*, and *rolC* modified genotypes. Error bars show the standard deviation for each set of samples. Error bars for HS samples are based on the mean relative error, as obtained from validation. Concentrations marked with an asterisk resulted statistically different at $p < 0.05$ according to the t-test.

HS is known to cause oxidative stress (Wang et al., 2010), generating reactive oxygen species (ROS) which compromise membrane fluidity, protein stability and enzymatic functions (Lipiec et al., 2013). To alleviate cellular damages and scavenge ROS compounds, plants produce antioxidant metabolites, such as flavonoids, anthocyanins, phenolics, whose biosynthesis starts from the SHA pathway (Lipiec et al., 2013; Obata and Fernie, 2012). Interestingly, in our study, SHA levels showed to increase only in wt-HS plants, indicating the activation of the shikimate metabolic route. This result is in agreement with the study of Ancillotti et al., 2015, who highlighted increased levels of total polyphenols and radical scavenging activity (RSA) in the shoots of wt-HS, while *gr*

and *rolC* plants showed reduced antioxidants levels. It is noteworthy that in wt-HS plants the levels of the precursor SHA were increased while SA, on the contrary, was reduced. This finding could suggest that the raised SHA produced in wt-HS plants is not employed for the biosynthesis of SA whereas, probably, it is mainly re-routed in the phenylpropanoid pathway for the synthesis of antioxidant compounds, in order to face ROS molecules. *RolC*-HS plants showed unchanged SHA levels, confirming the previous result of Bulgakov et al., 2008, who reported the prevention of oxidative burst in *Rubia cordifolia rolC* transformed cells and their increased HS tolerance. With respect to *gr* plants, the unchanged SHA levels could indicate that the induction of this metabolic pathway during HS is not required in this genotype; the higher basal levels of hydroxycinnamic acids, which are the main precursors of phenolics, suggest an enhancement of defenses in this genotype that, in cases of abiotic stress exposure, proved to be already active, not requiring an higher induction of the SHA pathway (Del Bubba et al., 2013).

3.3. Cr(VI) stress

Oxidative stress is one of the most evident consequences of heavy metal exposure through the enhanced production of ROS (Maksymiec, 2007). Particularly, exposure to Cr(VI) largely increases ROS levels, activating a signaling response at gene expression level which could increase active oxidant scavenging (Singh et al., 2015). In case of severe chemical stress, lipid peroxidation and the generation of oxylipins, such as JA, can take place (Maksymiec, 2007). In our samples, we actually observed an enhancement of JA and SHA levels in wt-Cr and *rolC*-Cr, with a statistically significant increase twice as high as in the controls (Fig. 2 b,c), while *gr*-Cr showed no significant changes. The induction of the octadecenoic pathway, probably caused by the degradation of membranes (Maksymiec et al., 2005), was already observed after exposure to heavy metals, leading to the production of JA and other oxylipins (Maksymiec, 2007; Maksymiec et al., 2005). This pathway is part of the oxidative/redox system of plants and, therefore, probably interacts with metal induced signaling and even in potential defense reactions (Viehweger, 2014). JA could be involved in the upregulation of the genes related to the metabolism of glutathione, which is a key element of cellular redox homeostasis and has a defense role against oxidative stress (Xiang and Oliver, 1998). As already outlined for plants exposed to HS, SHA represents an indicator of the induction of antioxidant biosynthesis. SHA proved to be indeed enhanced in wt-Cr and *rolC*-Cr samples, while it remained unchanged in *gr*-Cr, indicating a lower activation of the defense mechanisms in this genotype. Our results are in agreement with the recent study of Scalabrin et al., 2015 who reported increased levels of peroxylipids, lysolipids and antioxidant compounds in wt and *rolC* plants exposed to Cr(VI) stress while the levels of these metabolites were unchanged in the *gr* genotype. The SHA concentrations observed in this study are also consistent with the results of Fuoco et al., 2013 for wt and *gr* genotypes. The increase of SHA concentrations in *rolC*-Cr plants indicates that, differently from what is observed in *rolC*-HS, the ROS suppression effect is not effective against Cr(VI) stress. This fact is probably due to the complex action of heavy metals on the oxidative/redox system of plants, which includes the direct generation of ROS via Fenton-like reactions and the Haber−Weiss cycle, in addition to indirect mechanisms, and the consumption of glutathione as direct chelator for metal detoxification (Viehweger, 2014). This is also consistent with the enhanced concentration of JA in *rolC*-Cr samples. Interestingly, while in wt-Cr plants the increased SHA levels lead also to enhanced concentrations of its derivative, SA, in *rolC*-Cr plants only SHA was augmented. In wt-Cr plants, a significant SA increase of 57% was observed, in comparison to controls, while in *gr*-Cr and *rolC*-Cr SA levels were unchanged. The role of SA in heavy metal stress defense is currently not well understood; this compound showed to increase in Barley seedlings and maize plants subjected to high cadmium levels (Metwally et al., 2003; Pál et al., 2005) and in *Oryza sativa* spp. *Japonica* exposed to oxidative stress (Yang et al., 2004) while, in other studies, decreased or unchanged concentrations were observed (Singh et al., 2015). SA seems to increase oxidative stress by reducing the activity of phytochelatin synthase (Pál et al., 2002), increasing H_2O_2 levels (Zawoznik et al., 2007) and inhibiting the JA pathway (Maksymiec, 2007). The increase in SA concentrations could probably be considered as an early response to heavy metal stress, as already described for HS stress, which is needed in order to activate the plant response mechanisms (Zawoznik et al., 2007).

3.4. Water stress (WS)

In plants exposed to WS, a 50% decrease of SHA concentrations was observed in *gr*-Cr samples, in comparison to controls. The lower activation of SHA pathway in *gr*-WS plants than in *rolC*-WS and wt-WS genotypes was already reported (Scalabrin et al., 2015) and it's in accordance with the decreased RSA recently observed by Ancillotti et al., 2015. The plant's response to water deficit is complex and highly variable depending on the species, developmental stage, desiccation degree (Obata and Fernie, 2012), and interaction with other environmental factors, such as relative humidity and temperature. Different biochemical responses to water deficit conditions have even been observed in various varieties of the same species (Sánchez-Rodríguez et al., 2011). The shikimic acid pathway is generally induced by water stress exposition and different regulation mechanisms have been proposed, such as the maintenance of the osmotic pressure level (Warren et al., 2012), the need for increased antioxidant activity (Sánchez-Rodríguez et al., 2011) and the decreased lignification degree (Torras-Claveria et al., 2012). Therefore, the concentration of this compound could be affected by many factors and the comprehension of its role during WS still remains a challenge.

In our study, the decreased SHA level in *gr* genotype, seem to indicate a lower activation of plant defenses, compared to *rolc* and wt, which could be related to the higher resistance of *gr* plants toward WS; this suggestion, however, should be better investigated in order to assess the real potential of this genetic modification.

The role of SA and JA during WS conditions is currently under discussion. Our results suggested that the levels of these two phytohormone are not directly affected by water stress; however SA and JA could take part in the activation of early plant defenses, to be progressively depleted during stress exposure. Some studies indicate that high concentrations of SA could determine oxidative stress, thus leading to reduced abiotic stress tolerance, while, at moderate levels, SA is able to alleviate drought damages (Miura and Tada, 2014); in *A. thaliana* increased SA levels were related to an enhanced tolerance toward drought stress, as a consequence of the induction of H_2O_2 as signaling molecule (He et al., 2014). JA seems to be involved in ascorbate and glutathione metabolism, which is a key element in the cellular redox balance, and therefore could protect plants from water deficit-induced oxidative stress, as highlighted by malondialdehyde content and electrolyte leakage (Shan and Liang, 2010). Mahouachi et al., 2007 observed a transient accumulation of JA in papaya seedlings after 15 days of water deficit treatment, suggesting its role as a triggering signal for ABA accumulation.

The application of three independent abiotic stresses and the analysis of key compounds involved in stress-response permitted

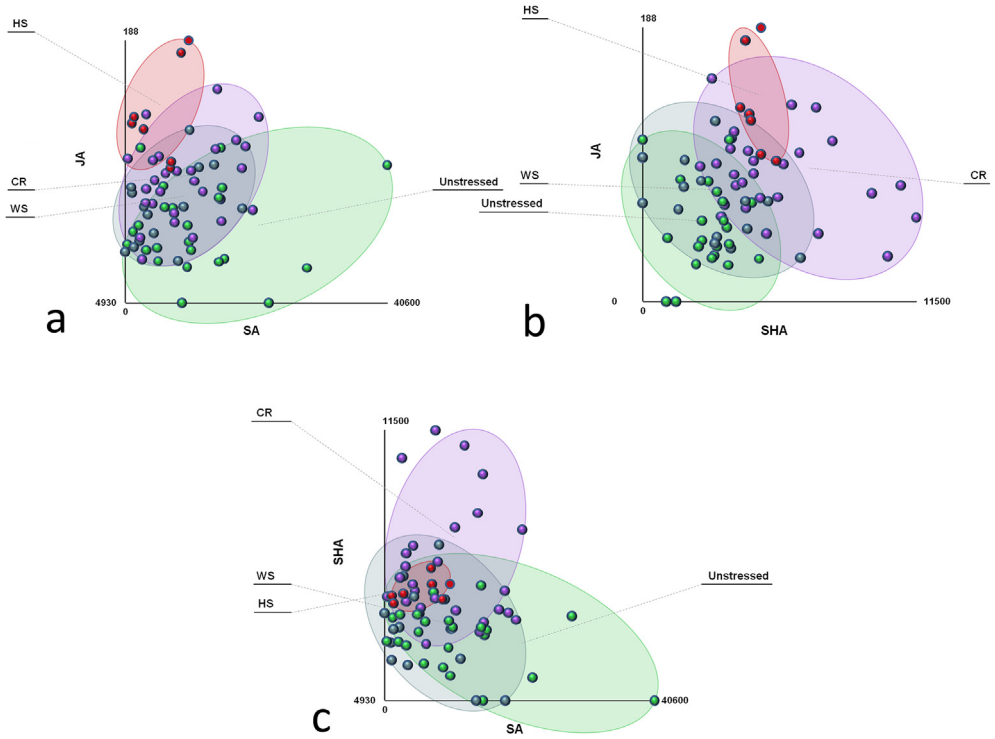

Fig. 3. a,b,c Scatterplots of phytohormone concentrations (a JA-SA, b JA-SHA, c SHA-SA) in samples classified by the kind of applied stress (controls: green, Cr stress: purple, HS stress: red, WS stress: gray). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to highlight the main metabolic re-routing associated with each stress; as summarized in Fig. 3 a,b,c, Cr samples were characterized by medium–high concentrations of SHA, a clear indication of the enhanced production of secondary metabolites and particularly antioxidants; HS samples, instead, showed particularly high levels of JA (Fig 3 a,b), probably as a consequence of the peroxidation of lipids and the activation of the octadecenoic pathway induced by heat; WS samples displayed metabolite concentrations generally similar to the controls, indicating that the water deficit conditions involves different defense mechanisms and, probably, a more complex plant response.

4. Conclusions

The method presented in this work enabled the simultaneous and sensitive determination of three relevant plant metabolites, which proved to be useful in the assessment of the plant metabolic and biochemical status. The investigation of wild and genetically modified plants, exposed to three abiotic stresses, allowed the evaluation of the combined effect of transgenes and stresses on plant response mechanisms. The three genotypes showed to induce different responses during stress exposure; particularly, the insertion of the *gr* transgene showed to determine less changes in the phytohormonal pattern, suggesting the possibility of an advantage deriving from this genetic modification, especially in case of WS and Cr stresses, as already proposed in previous studies. These results could be useful for further investigations concerning the use of transgenic-stress resistant plants and in order to better understand the role of SA, JA and SHA in plant defense mechanisms.

Contributions

Elisa Scalabrin conducted the experiments, analyzed the data and wrote most of the manuscript. Marta Radaelli helped in plant sample preparation and revised the manuscript. Gabriele Capodaglio conceived and designed the experiments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.02.040.

References

Ancillotti, C., Bogani, P., Biricolti, S., Calistri, E., Checchini, L., Ciofi, L., Gonnelli, C., Del Bubba, M., 2015. Changes in polyphenol and sugar concentrations in wild type and genetically modified *Nicotiana langsdorffii* Weinmann in response to water and heat stress. Plant Physiol. Biochem. 97, 52–61. http://dx.doi.org/10.1016/j.plaphy.2015.09.012.

Bartwal, A., Mall, R., Lohani, P., Guru, S.K., Arora, S., 2013. Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. J. Plant Growth Regul. 32, 216–232. http://dx.doi.org/10.1007/s00344-012-9272-x.

Bogani, P., Calistri, E., Biricolti, S., Ruffini, M., Turrini, A., Giovannetti, M., Buiatti, M., 2015. The rat glucocorticoid receptor integration in *Nicotiana langsdorffii* genome affects plant responses to abiotic stresses and to arbuscular mycorrhizal symbiosis. Plant Biotechnol. Rep. 9, 209–220. http://dx.doi.org/10.1007/s11816-015-0358-3.

Bulgakov, V.P., Aminin, D.L., Shkryl, Y.N., Gorpenchenko, T.Y., Veremeichik, G.N., Dmitrenok, P.S., Zhuravlev, Y.N., 2008. Suppression of reactive oxygen species and enhanced stress tolerance in *Rubia cordifolia* cells expressing the rolc oncogene. Mol. Plant. Microbe. Interact. 21, 1561–1570. http://dx.doi.org/10.1094/MPMI-21-12-1561.

Clarke, S.M., Cristescu, S.M., Miersch, O., Harren, F.J.M., Wasternack, C., Mur, L.A., 2009. Jasmonates act with salicylic acid to confer basal thermotolerance in

Arabidopsis thaliana. New Phytol. 182, 175–187. http://dx.doi.org/10.1111/j.1469-8137.2008.02735.x.

De Ollas, C., Hernando, B., Arbona, V., Gómez-Cadenas, A., 2013. Jasmonic acid transient accumulation is needed for abscisic acid increase in citrus roots under drought stress conditions. Physiol. Plant 147, 296–306. http://dx.doi.org/10.1111/j.1399-3054.2012.01659.x.

Del Bubba, M., Ancillotti, C., Checchini, L., Ciofi, L., Fibbi, D., Gonnelli, C., Mosti, S., 2013. Chromium accumulation and changes in plant growth, selected phenolics and sugars of wild type and genetically modified *Nicotiana langsdorffii*. J. Hazard. Mater 262, 394–403. http://dx.doi.org/10.1016/j.jhazmat.2013.08.073.

Fuoco, R., Bogani, P., Capodaglio, G., Del Bubba, M., Abollino, O., Giannarelli, S., Spiriti, M.M., Muscatello, B., Doumett, S., Turetta, C., Zangrando, R., Zelano, V., Buiatti, M., 2013. Response to metal stress of *Nicotiana langsdorffii* plants wild-type and transgenic for the rat glucocorticoid receptor gene. J. Plant Physiol. 170, 668–675. http://dx.doi.org/10.1016/j.jplph.2012.12.009.

Giannarelli, S., Muscatello, B., Bogani, P., Spiriti, M.M., Buiatti, M., Fuoco, R., 2010. Comparative determination of some phytohormones in wild-type and genetically modified plants by gas chromatography-mass spectrometry and high-performance liquid chromatography-tandem mass spectrometry. Anal. Biochem. 398, 60–68. http://dx.doi.org/10.1016/j.ab.2009.10.038.

He, Q., Zhao, S., Ma, Q., Zhang, Y., Huang, L., Li, G., Hao, L., 2014. Endogenous salicylic acid levels and signaling positively regulate Arabidopsis response to polyethylene glycol-simulated drought stress. J. Plant Growth Regul. 33, 871–880. http://dx.doi.org/10.1007/s00344-014-9438-9.

Intrieri, M.C., Buiatti, M., 2001. The horizontal transfer of *Agrobacterium rhizogenes* genes and the evolution of the genus Nicotiana. Mol. Phylogenet. Evol. 20, 100–110. http://dx.doi.org/10.1006/mpev.2001.0927.

Kazan, K., 2015. Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Sci. 20, 219–229. http://dx.doi.org/10.1016/j.tplants.2015.02.001.

Khalid, K.a., Teixeira, J.a., Cai, W., 2010. Water deficit and polyethylene glycol 6000 affects morphological and biochemical characters of *Pelargonium odoratissimum* (L.). Sci. Hortic. Amst. 125, 159–166. http://dx.doi.org/10.1016/j.scienta.2010.03.009.

Kiselev, K.V., Kusaykin, M.I., Dubrovina, A.S., Bezverbny, D.A., Zvyagintseva, T.N., Bulgakov, V.P., 2006. The rolC gene induces expression of a pathogenesis-related beta-1,3-glucanase in transformed ginseng cells. Phytochemistry 67, 2225–2231. http://dx.doi.org/10.1016/j.phytochem.2006.07.019.

Kurepin, L.V., Dahal, K.P., Zhaman, M., Pharis, R.P., 2013. Interplay between Environmental Signals and Endogenous Salicylic Acid Concentration. In: Hayat, Shamsul, Ahmad, Aqil, Alyemeni, M.N. (Eds.), Salicylic Acid:Plant Growth and Development. Springer Science & Business Media, pp. 61–82.

Lipiec, J., Doussan, C., Nosalewicz, A., Kondracka, K., 2013. Effect of drought and heat stresses on plant growth and yield: a review. Int. Agrophysics 27, 463–477. http://dx.doi.org/10.2478/intag-2013-0017.

Mahouachi, J., Arbona, V., Gómez-Cadenas, A., 2007. Hormonal changes in papaya seedlings subjected to progressive water stress and re-watering. Plant Growth Regul. 53, 43–51. http://dx.doi.org/10.1007/s10725-007-9202-2.

Maksymiec, W., 2007. Signaling responses in plants to heavy metal stress. Acta Physiol. Plant 29, 177–187. http://dx.doi.org/10.1007/s11738-007-0036-3.

Maksymiec, W., Wianowska, D., Dawidowicz, A.L., Radkiewicz, S., Mardarowicz, M., Krupa, Z., 2005. The level of jasmonic acid in *Arabidopsis thaliana* and Phaseolus coccineus plants under heavy metal stress. J. Plant Physiol. 162, 1338–1346. http://dx.doi.org/10.1016/j.jplph.2005.01.013.

Metwally, A., Finkemeier, I., Georgi, M., Dietz, K., 2003. Salicylic acid alleviates the cadmium toxicity in Barley seedlings. Plant Physiol. 132, 272–281. http://dx.doi.org/10.1104/pp.102.018457.

Miura, K., Tada, Y., 2014. Regulation of water, salinity, and cold stress responses by salicylic acid. Front. Plant Sci. 5, 1–12. http://dx.doi.org/10.3389/fpls.2014.00004.

Obata, T., Fernie, A.R., 2012. The use of metabolomics to dissect plant responses to abiotic stresses. Cell. Mol. life Sci. 69, 3225–3243. http://dx.doi.org/10.1007/s00018-012-1091-5.

Pál, M., Horváth, E., Janda, T., Páldi, E., Szalai, G., 2005. Cadmium stimulates the accumulation of salicylic acid and its putative precursors in maize (Zea mays) plants. Physiol. Plant 125, 356–364. http://dx.doi.org/10.1111/j.1399-3054.2005.00545.x.

Pál, M., Szalai, G., Horváth, E., Janda, T., Páldi, E., 2002. Effect of salicylic acid during heavy metal stress. Acta Biol. szeg. 119–120.

Pan, Q., Zhan, J., Liu, H., Zhang, J., Chen, J., Wen, P., Huang, W., 2006. Salicylic acid synthesized by benzoic acid 2-hydroxylase participates in the development of thermotolerance in pea plants. Plant Sci. 171, 226–233. http://dx.doi.org/10.1016/j.plantsci.2006.03.012.

Ranaldo, M., Toscano, G., Radaelli, M., Scalabrin, E., Capodaglio, G., 2015. *Nicotiana langsdorffii* wild type and genetically modified exposed to chemical and physical stress: changes in element content. Int. J. Environ. Anal. Chem. 95, 349–365. http://dx.doi.org/10.1080/03067319.2015.1016013.

Rizhsky, L., Liang, H., Mittler, R., 2002. The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol. 130, 1143–1151. http://dx.doi.org/10.1104/pp.006858.

Sánchez-Rodríguez, E., Moreno, D.a., Ferreres, F., Rubio-Wilhelmi, M.D.M., Ruiz, J.M., 2011. Differential responses of five cherry tomato varieties to water stress: changes on phenolic metabolites and related enzymes. Phytochemistry 72, 723–729. http://dx.doi.org/10.1016/j.phytochem.2011.02.011.

Sano, H., Seo, S., Koizumi, N., Niki, T., Iwamura, H., Ohashi, Y., 1996. Regulation by cytokinins of endogenous levels of jasmonic and salicylic acids in mechanically wounded tobacco plants. Plant Cell Physiol. 37, 762–769. http://dx.doi.org/10.1093/oxfordjournals.pcp.a029011.

Scalabrin, E., Radaelli, M., Rizzato, G., Bogani, P., Buiatti, M., Gambaro, A., Capodaglio, G., 2015. Metabolomic analysis of wild and transgenic *Nicotiana langsdorffii* plants exposed to abiotic stresses: unraveling metabolic responses. Anal. Bioanal. Chem. 407, 6357–6368. http://dx.doi.org/10.1007/s00216-015-8770-7.

Schmelz, E.A., Engelberth, J., Alborn, H.T., O' Donnell, P., Sammons, M., Toshima, H., Tumlinson III, J.H., 2003. Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. Proc. Natl. Acad. Sci. U. S. A. 100, 10552–10557. http://dx.doi.org/10.1073/pnas.1633615100.

Shan, C., Liang, Z., 2010. Jasmonic acid regulates ascorbate and glutathione metabolism in *Agropyron cristatum* leaves under water stress. Plant Sci. 178, 130–139. http://dx.doi.org/10.1016/j.plantsci.2009.11.002.

Singh, V.P., Kumar, J., Singh, M., Singh, S., Prasad, S.M., Dwivedi, R., Singh, M.P.V.V.B., 2015. Role of salicylic acid-seed priming in the regulation of chromium (VI) and UV-B toxicity in maize seedlings. Plant Growth Regul. http://dx.doi.org/10.1007/s10725-015-0076-4.

Torras-Claveria, L., Jáuregui, O., Codina, C., Tiburcio, A.F., Bastida, J., Viladomat, F., 2012. Analysis of phenolic compounds by high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry in senescent and water-stressed tobacco. Plant Sci. 182, 71–78. http://dx.doi.org/10.1016/j.plantsci.2011.02.009.

Viehweger, K., 2014. How plants cope with heavy metals. Bot. Stud. 55, 35. http://dx.doi.org/10.1186/1999-3110-55-35.

Wang, L.-J., Fan, L., Loescher, W., Duan, W., Liu, G.-J., Cheng, J.-S., Luo, H.-B., Li, S.-H., 2010. Salicylic acid alleviates decreases in photosynthesis under heat stress and accelerates recovery in grapevine leaves. BMC Plant Biol. 10, 34. http://dx.doi.org/10.1186/1471-2229-10-34.

Warren, C.R., Aranda, I., Cano, F.J., 2012. Metabolomics demonstrates divergent responses of two Eucalyptus species to water stress. Metabolomics 8, 186–200. http://dx.doi.org/10.1007/s11306-011-0299-y.

Xiang, C., Oliver, D.J., 1998. Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis. Plant Cell 10, 1539–1550. http://dx.doi.org/10.1105/tpc.10.9.1539.

Yang, Y., Qi, M., Mei, C., 2004. Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. Plant J. 40, 909–919. http://dx.doi.org/10.1111/j.1365-313X.2004.02267.x.

Yordanov, I., Velikova, V., Tsonev, T., 2000. Plant responses to drought, acclimation and stress tolerance. Photosynthetica 38, 171–186. http://dx.doi.org/10.1023/A:1007201411474.

Zawoznik, M.S., Groppa, M.D., Tomaro, M.L., Benavides, M.P., 2007. Endogenous salicylic acid potentiates cadmium-induced oxidative stress in *Arabidopsis thaliana*. Plant Sci. 173, 190–197. http://dx.doi.org/10.1016/j.plantsci.2007.05.004.