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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).