Journal of Plant Physiology 231 (2018) 261–270

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Effect of heat root stress and high salinity on glucosinolates metabolism in wild rocket

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ARTICLE INFO

Keywords:
Abiotic stress
Bioactive compounds
Chlorophyll fluorescence
Diplotaxis tenuifolia
Gene expression
Nutraceuticals

ABSTRACT

Wild rocket (*Diplotaxis tenuifolia* L.) is a leafy vegetable appreciated for its characteristic sensory properties which are mainly due to the presence of glucosinolates (GSLs). Short-term exposure to abiotic stresses can induce physiological responses and transcriptional changes which involve GSLs. For this reason, the aim of this work was to study the mechanisms of regulation of GSLs metabolism in rocket subjected to heat stress (40 °C) and high salinity (200 mM NaCl) imposed for up to 48 h. GSLs levels and the expression of methylthioalkylmalate synthase1 (*DtMAM1*), cytochromeP79F1 (*DtCYP79F1*), cytochromeP45083A1 (*DtCYP83A1*), cytosolic-sulfotransferase5b (*DtST5b*), cytosolic-sulfotransferase5c (*DtST5c*), flavinmono-oxygenase (*DtFMO*), myrosinase (*DtMyro*) and thio-methyl transferase (*DtTMT*) were analyzed under stress conditions. In addition, the effect on chlorophyll and glucose levels, as well as on chlorophyll *a* fluorescence were evaluated. Chlorophyll and chlorophyll fluorescence were not affected by the short-term application of stresses. Glucose levels in roots were doubled in response to high salinity, while, in the same organ, GSLs were three fold lower in response to both stresses. The relative content of several aliphatic GSLs was significantly reduced in leaves as a response to both stresses. A key role in GSLs metabolism and in the response to salinity is hypothesized for the gene *DtTMT*, as it showed an increment in transcripts accumulation (three-fold) consistent with the decrement in the GSLs levels found in salt-exposed leaves and roots. The results obtained in this study can be used in breeding programmes aiming to enhance rocket sensory quality and to improve the resistance to abiotic stresses.

1. Introduction

In the recent years, the commercial relevance of wild rocket (*Diplotaxis tenuifolia*, L.) has largely increased. This leafy vegetable, which belongs to the family of *Brassicaceae*, is appreciated for its peculiar sensory attributes and at the same time, is a good source of phytonutrients (Bell and Wagstaff, 2014), including glucosinolates (GSLs). GSLs are β-thioglucoside N-hydrosulfates responsible for the sharp and bitter-tasting flavors found exclusively in the order *Capparales*, which contains 15 families, including *Brassicaceae* (Halkier and Gershenzon, 2006; Gupta et al., 2012). Around 200 structurally different GSLs were identified, and they can be classified as aliphatic, indole or aromatic based on their primary precursor amino acids (Sønderby et al., 2007). Biosynthesis of all GSLs involves mainly three steps, namely, elongation of amino acid chain, biosynthesis of core structure (aglycone) and secondary modification of amino acid side chains (Wang et al., 2011). This metabolic route has been characterized in several species and the key genes involved in the GSLs biosynthesis have been identified (Sønderby et al., 2010; Wang et al., 2011). GSLs coexist with their degradative enzyme thioglucoside glucohydrolase (E.C. 3.2.1.147), also known as myrosinase. Myrosinase is present in myrosin cells in plants and in the human intestinal flora (Bennett et al., 2006). The enzymatic hydrolysis of GSLs catalyzed by myrosinase lead to the formation of isothiocyanates (ITCs), thiocyanates, and epithionitriles (Bones et al., 2015). Mechanical injuries can cause tissue disruption and plant cell breakage. These damages can lead to decompartmentalization between plant vacuole and myrosin cells and finally to the breakdown of GSLs. After the degradation of GSLs, the enzyme thio-methyl transferase (E.C. 2.1.1.9) catalyzes the modification of the GSLs hydrolytic products (Attieh et al., 2002). During cultivation high temperature and high salinity can affect roots as well as shoot and can reduce crops quality and yield. Rocket is often grown as a baby leaf

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https://doi.org/10.1016/j.jplph.2018.10.003
Received 10 August 2018; Received in revised form 1 October 2018; Accepted 2 October 2018
Available online 06 October 2018

crop in hydroponic system or in coastal areas characterized by high soil salinity. These stresses are a common problem in Mediterranean countries, also due to the possible heating of the nutrient solution and to the low quality (high salinity) of the water used for the cultivation. Roots represent the primary fence against soil adverse conditions and can modulate molecular and physiological responses also in the aerial part of the plant by regulating the whole-plant carbon and water relations (Aidoo et al., 2016). Abiotic stresses can thus affect plant metabolism and lead to important product losses (up to 70%, Boyer, 1982; Mariani and Ferrante, 2017). At the same time, abiotic stresses can induce specific responses at cellular level, which can help in counteracting the stressful conditions. These responses are various, and in certain cases can involve the *de novo* biosynthesis of secondary metabolites as well as their degradation or structural modifications. Several studies indicate that environmental factors may modify GSLs and isothiocyanates plant composition, but their physiological role in response to abiotic stresses is not completely known yet (Martínez-Ballesta et al., 2013). The aim of this work was to generate information regarding GSLs pathway regulation in wild rocket under environmental stresses and to understand the molecular processes which can drive the plant response to short-time controlled stress applications in both leaves and roots. To achieve these goals, the changes in total GSLs and the expression of some of the genes involved in GSLs metabolism were measured in response to heat root stress and high salinity. HPLC-ESI/MS analysis was performed for identifying the GSLs contained in rocket leaves and to estimate their changes in response to stresses. In addition, to assess the response of plant primary metabolism, chlorophyll and chlorophyll *a* fluorescence indexes, together with glucose, were also measured.

2. Materials and methods

2.1. Plant material

Wild rocket (*Diplotaxis tenuifolia* cv Frastagliata, ISI Sementi, SpA, Italy) seeds were sown on December the 17th 2015, in trays (51.5 x 32.5 cm) with 228 holes filled with perlite. Expected plant density was 1150 plants m^{-2}. Oxygen was supplied by bubbling air in the nutrient solution and keeping the oxygen concentration around 5–6 mg L^{-1}. Cultivation took place in an experimental greenhouse under monitored growing conditions. An optimized Hoagland's solution was used as plant growing media. The concentrations (expressed as mM) of nutrients in the solution were: 12 N-NO_3, 3.8 N-NH_4, 2.8 P, 8.4 K, 3.5 Ca, 1.4 Mg and Hoagland's concentration for micronutrients.

2.2. Heat and salinity stress treatments

For heat root stress treatment, plants were transferred to a fresh nutrient solution constantly maintained at 40 °C in a water bath equipped with thermostat control. For salinity stress, plants were transferred to a fresh nutrient solution containing 200 mM NaCl (EC: 19 dS/m). The nutrient solution of control plants was also changed. Fresh nutrient solutions were prepared on February the 1st, while plants were transferred on February the 2nd 2016 at 8:00 (47 days after sowing). For gene expression analyses, samples of 0.25 g (leaves and roots) were collected from four plants at 10:00, 12:00, 14:00, 17:00 and 8:00 (after 2, 4, 6, 9, and 24 h of exposure to heat stress, salt stress as well as from non-stressed control). Complete harvesting of roots and leaves was also done on February the 4th (after 48 h of stress) and the material collected was further used for the determination of glucose and for GSLs analyses. All samples were snap frozen in liquid nitrogen and immediately transferred to −80 °C to prevent wounding and RNA degradation.

2.3. Non-destructive determinations

2.3.1. Chlorophyll content

in vivo chlorophyll content was estimated 24 and 48 h after treatment for each stress as well as of control. CL-01Chlorophyll Content System (Hansatech, Kings Lynn, UK) was used for measuring chlorophyll levels. The adaxial side of the leaf was consistently placed towards the emitting window of the equipment avoiding the major veins. For each treatment, six leaves from six different plants were read.

2.3.2. Chlorophyll a Fluorescence

in vivo chlorophyll *a* fluorescence was measured 24 and 48 h after treatment for each stress as well as for control using a hand-portable fluorometer (Handy PEA, Hansatech, Kings Lynn, UK). Chlorophyll *a* fluorescence was measured on dark adapted leaves, kept for 30 min at room temperature. Measurements were taken on the leaf surface (4 mm diameter) exposed to an excitation light intensity [ultra-bright red light emitting devices (LEDs) with a peak at 650 nm] of 3000 µmol m^{-2} s^{-1} (600 Wm^{-2}) emitted by three diodes. Fluorescence detection was measured by fast response PIN photodiode with RG9 long pass filter (Technical manual, Hansatech, Kings Lynn, UK).

2.4. Destructive determinations

2.4.1. Glucose and total glucosinolates

The method described by Hsu et al. (2011) with minor modifications was used for the estimation of glucose and total GSLs from leaves and roots samples of rocket. According to this method the moles of glucose derived from GSLs hydrolysis can be used to calculate the total amount of GSLs that have been hydrolyzed by the action of the endogenous myrosinase. One hundred milligrams of frozen leaves or roots were ground in liquid nitrogen, then, in a set of samples, 280 mL of acidified methanol (40% methanol and 0.5% acetic acid) were added to prevent GSLs hydrolysis by endogenous myrosinase. In another set of samples, 280 mL of distilled H_2O were added, and the mixture was incubated at 37 °C for 10 min to allow the complete hydrolysis of GSLs. Two hundred and ten milliliters of 100% methanol were added to stop the reaction and 2 mg of activated carbon were added to precipitate polyphenols. All samples were then centrifuged twice at 13 000 rpm, 4 °C for 10 min and the extract obtained was used for the determination of glucose. The R-Biopharm kit (Boehringer, Mannheim, Germany) was used for the determination of glucose in both extracts and the total GSLs content was thus calculated as the difference between the glucose content in the hydrolyzed sample and the glucose content in the control sample not subjected to hydrolysis.

2.4.2. Glucosinolates analysis by HPLC-ESI/MS

Glucosinolate components have been identified in leaves extracts by HPLC coupled to mass spectrometry with ElectroSpray Ionization (ESI). Analysis was performed on an PE Sciex API 365 triple quadrupole mass spectrometer (Sciex, Concord, Ontario, Canada) coupled to an Agilent G1312 A binary pump with high pressure solvent mixing, equipped with an Agilent G1329 A autosampler (Agilent, Santa Clara, California, USA).

HPLC separation was carried out on a Phenomenex Kinetex EVO C18 100 x 2.1 mm, 5 µm particle size column (Phenomenex, Torrance, California, USA) using a gradient water/acetonitrile with 0.1% formic acid at 300 µl min^{-1}.

Samples were analyzed in scan mode (200–1000 Th) both in positive and negative ion mode under the following experimental conditions:

Nebulizing Gas, NEB, 8 (arbitrary units); Curtain Gas, CUR, 8 (arbitrary units); Temperature, TEM, 350 °C; Nebulizer Voltage, IS, 5000 V (-4200 V in negative ion mode); Declustering Potential, DP; 10 V (-10 V in negative ion mode); Focusing Potential, FP, 200 V (-200 V in negative ion mode), Scan Rate, 200–1000 Th in 3 s.