Plant Physiology and Biochemistry 109 (2016) 452–466

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Glucosinolate biosynthesis in *Eruca sativa*

Dimitra Katsarou [a], Michalis Omirou [b], Kalliopi Liadaki [a], Daniela Tsikou [a], Costas Delis [a, 1], Constantine Garagounis [a], Afrodite Krokida [a], Antonis Zambounis [a], Kalliope K. Papadopoulou [a, *]

[a] University of Thessaly, Department of Biochemistry & Biotechnology, Larisa, Greece
[b] Agricultural Research Institute, Ministry of Agriculture, Natural Resources and Environment, Nicosia, Cyprus

ARTICLE INFO

Article history:
Received 4 August 2016
Received in revised form
6 October 2016
Accepted 25 October 2016
Available online 26 October 2016

Keywords:
Glucosinolates
Biosynthesis
Cancer cells
Cytotoxicity
Plant nutrition
Brassicaceae

ABSTRACT

Glucosinolates (GSLs) are a highly important group of secondary metabolites in the Caparalles order, both due to their significance in plant-biome interactions and to their chemoprotective properties. This study identified genes involved in all steps of aliphatic and indolic GSL biosynthesis in *Eruca sativa*, a cultivated plant closely related to *Arabidopsis thaliana* with agronomic and nutritional value. The impact of nitrogen (N) and sulfur (S) availability on GSL biosynthetic pathways at a transcriptional level, and on the final GSL content of plant leaf and root tissues, was investigated. N and S supply had a significant and interactive effect on the GSL content of leaves, in a structure-specific and tissue-dependent manner; the metabolites levels were significantly correlated with the relative expression of the genes involved in their biosynthesis. A more complex effect was observed in roots, where aliphatic and indolic GSLs and related biosynthetic genes responded differently to the various nutritional treatments suggesting that nitrogen and sulfur availability are important factors that control plant GSL content at a transcriptional level. The biological activity of extracts derived from these plants grown under the specific nutritional schemes was examined. N and S availability were found to significantly affect the cytotoxicity of *E. sativa* extracts on human cancer cells, supporting the notion that carefully designed nutritional schemes can promote the accumulation of chemoprotective substances in edible plants.

1. Introduction

Eruca sativa is an edible plant indigenous to the Mediterranean, and was traditionally used for its medicinal properties. The plant has a high vitamin C content and is known for various health-promoting effects, including improvement of blood circulation, diuretic and anti-inflammatory properties (Bennett et al., 2006; Fuentes et al., 2014; Khoobchandani et al., 2010). The increase in cultivation of *E. sativa* is due to its promotion as a fourth generation salad plant and additionally to superior yields of high value-added products, the most prominent being erucic acid (Yaniv et al., 1998). *E. sativa* is a diploid plant of the Brassicaceae family, with a short life-cycle (6–9 weeks) and a relatively small genome (~560 Mb) that renders it suitable for molecular studies.

Similar to other *Brassica* species, the characteristic pungent taste and odor of *E. sativa* leaves is attributed to their glucosinolate content. Glucosinolates (GSLs) are viewed as one of the most important groups of plant secondary metabolites, with more than 120 intact GSLs having been isolated and characterized, exclusively in the Caparrales order (Fahey et al., 2001). Myrosinase-dependent hydrolysis, which takes place after tissue disruption, results in the formation of a variety of different GSL-derived compounds. Intensive research during the last decade has revealed that GSLs and their degradation products have chemo-preventive, anticancer properties (Ioannides and Konsue, 2015; Khoobchandani et al., 2011; Melchini et al., 2009). For instance, sulforaphane, the hydrolysis product of the GSL glucoraphanin, has been recognized as one of the most potent anti-carcinogenic compounds in nature (Traka et al., 2014). Additional evidence suggests that intact GSLs can modulate carcinogen-metabolizing enzyme activities (Abdull Razis et al., 2010, 2011), thus increasing interest in identifying, or generating, plants with high GSL content.

GSLs are divided into three major categories: aliphatic, indolic and aromatic, depending on their precursor compounds. Aliphatic

* Corresponding author. University of Thessaly, Department of Biochemistry & Biotechnology, Biopolis, 41500, Larissa, Greece.
E-mail address: kalpapad@bio.uth.gr (K.K. Papadopoulou).
[1] Present Address: TEI Peloponnese, Kalamata, Greece.

http://dx.doi.org/10.1016/j.plaphy.2016.10.024
0981-9428/

GSLs are derived mainly from methionine; indolic GSLs from tryptophan and aromatic GSLs from phenylalanine. Extensive research in the model plant *Arabidopsis thaliana* revealed the GSL biosynthesis pathways through gene identification and isolation of their expression products. Glucosinolate biosynthesis occurs in three successive steps: 1) amino acid elongation for aliphatic GSLs, 2) core structure formation and 3) side chain modification. In the first step of GSL biosynthesis, methionine is de-aminated to a 2-oxo-acid, which in turn is condensed with acetyl-CoA. A subsequent de-carboxylation step leads to the net gain of one methyl group, and this elongation cycle can be repeated two to six times (de Kraker and Gershenzon, 2011). These reactions are catalyzed by enzymes belonging to the methylthioalkylmalate synthases family (MAMs), members of which have been isolated from *E. sativa* (Graser et al., 2000). In the second step, the resulting chain-elongated products are oxidized to the corresponding aldoximes by cytochrome P_{450} enzymes, namely CYP79F1 and CYP79F2. During indolic GSL biosynthesis, tryptophan is directly converted to the corresponding aldoximes by CYP79B2 and CYP79B3. The aldoxime precursors of both aliphatic and indolic GSLS are then metabolized to S-alkylthiohydroximate conjugates by CYP83A1 and CYP83B1, respectively (Mikkelsen et al., 2002). Both enzymes metabolize aromatic oximes derived from phenylalanine, tryptophan, and tyrosine, although CYP83B1 has higher affinity for these substrates than CYP83A1 (Naur et al., 2003). A C-S lyase (SUR1) catalyzes the cleavage of the S-alkylthiohydroximates and the formation of thiohydroximates (Mikkelsen et al., 2004) Subsequently, and depending on the origin of each thiohydroximate, glucosyltransferases catalyse the glucosylation of thiohydroximates and the formation of desulfo-glucosinolates. All thiohydroximates are glucosylated by UTG74B1, while UGT74C1 plays a distinctive role in the synthesis of aliphatic glucosinolates (Grubb et al., 2014). The core structure formation is completed by the sulfation reactions of desulfo-GSLs that are catalyzed by three substrate specific sulfotransferases (Piotrowski et al., 2004). The final step of GSL biosynthesis involves the side chain modification reactions, which are responsible for the differences in biological activity of the intact and myrosinase-hydrolyzed GSL byproducts found in plants (Kliebenstein et al., 2001). Flavin monooxygenases are among the enzymes involved in side chain modification reactions of aliphatic GSLs and, specifically, in the oxidation of methylthioalkyl-GSLs into methylsulfinylalkyl GSLs (Hansen et al., 2007). Transcriptional regulation of GSL biosynthetic genes is reportedly under the control of MYB-family transcription factors (Sonderby et al., 2010a). In *A. thaliana,* transcript levels of GSL biosynthetic genes were strongly suppressed in MYB-knockout mutants, and GSL content was simultaneously reduced (Gigolashvili et al., 2009). To date there is no information on the genes involved in biosynthesis of GSL in *E. sativa*.

The type and concentration of individual GSLs in plants is determined both by abiotic and biotic factors. Genetic variability, pathogen and insect infection alters GSL content in plant tissues qualitatively and quantitatively. Glucosinolate metabolism is highly affected by nitrogen (N) and sulfur (S) availability and several studies demonstrated that N and S fertilization strategies are of primary importance for GSL content. Reduction of S supply resulted in a significant reduction of the GSL content of several Brassica species (Kopsell et al., 2007; Omirou et al., 2009). The impact of N on GSL concentration seems to be interrelated with S availability and this is mainly attributed to the plant genotype and growth stage; in addition, different types of GSLs respond differentially to the various fertilization schemes (Omirou et al., 2009; Schonhof et al., 2007). We previously reported that, increased N supply resulted in a reduction of most aliphatic GSLs but an in increase in indolic GSLs in *E. sativa* (Omirou et al., 2012). N and S fertilization schemes may affect the GSL biosynthetic machinery at a transcriptional level, but the available data are limited (Hirai et al., 2004; Maruyama-Nakashita et al., 2006).

This study examines the impact of long-term N and S starvation on the GSL content and transcript levels of biosynthetic genes and transcription factors involved in regulation of GSL biosynthesis in *E. sativa*. A factorial experimental design was employed, and both root and leaf tissues were sampled and analyzed. An additional objective was to evaluate the bioactivity of the extracts derived from tissues of plants grown under different N and S nutritional schemes, specifically the cytotoxic activity on human cancer cells, with the ultimate aim to understand how the effects of N and S fertilization on plant GSL content can translate into improved crop quality with benefits for human health.

2. Materials and methods

2.1. Plant material and experimental design

A local variety of *E. sativa* Mills was used. Seed is propagated and maintained in the Agricultural Research Institute in Nicosia under the code ACC10. Seeds were germinated on sterile, moist filter paper. Eighteen 5- days old seedlings per treatment were transferred to pots containing perlite and grown under two different nitrogen (N) and sulfur (S) nutritional schemes, in a completely randomized, factorial design. Each treatment was replicated 3 times. Plants were irrigated using modified nutrient solution, consisting of the following: KNO_3 7.13 mM, $MgSO_4{}^{7H}{}_2O$ 1 mM, KH_2PO_4 1 mM, $Ca(NO_3)_2{}^{4H}{}_2O$ 0.5 mM and Fe (EDTA) 0.1 mM. Nitrogen and S deficient conditions were achieved by replacing KNO_3 and $MgSO_4 \cdot 7H2O$ with KCl 7.11 mM and $MgCl_2 \cdot 6H2O$ 1 mM, respectively. Micronutrients were supplied by mixing 1 ml of a nutrient solution consisting of H_3BO_3 2.86 g/L, $MnCl_2{}^{4H}{}_2O$ 1.81 g/L, $ZnSO_4 \cdot H_2O$ 0.11 g/L, $CuCl_2{}^{2H2O}$ 0.05 g/L, $Na_2MoO_4 2H_2O$ 0.027 g/L in 1 L of water. Thus, under the four nutritional combinations employed, plants grown under optimal conditions (+N/+S) received 114 mg N/L and 32 mg S/L while plants grown under N and S deficiency conditions received 14 mg N/L and 0.019 mg S/L, which represent a severe deficiency for the S demanding cruciferous plant. Before the initiation of the experiment, seedlings were acclimated by irrigating only with deionized water for 3 days. Plants were grown in a growth chamber at 23 °C with a photoperiod of 16/8 h light/dark cycle, at 200 μmol photons m^{-2} s^{-1}. Samples were taken 35 days after the initiation of the experiment and before flowering. Plants were carefully removed from the pots, fresh weight of shoots and roots were recorded and immediately frozen in liquid nitrogen. Intact and undamaged plant leaves were stored at −80 °C before RNA extraction and freeze-drying for GSL analysis and cell culture bioassays.

2.2. Glucosinolates analysis

Freeze-dried tissues from roots and shoots were ground and GSLs were extracted as described previously based on the official ISO method (Omirou et al., 2012). In detail, 300 mg of the freeze dried grounded material was transferred to a 50 ml polypropylene conical tube with 5 ml EtOH 70% (70 °C), 150 μl sinigrin (6.65 mM) as an internal standard and 250 μl Tris (2-carboxyethyl) phosphine (TCEP) and homogenized with a U-Turrax at 14,500 rpm (T18, IKA Germany) for 3 min. TCEP was used in order to avoid dimer formation of glucosativin (Bennett et al., 2007). To test the impact of TCEP on GSLs profile, non-TCEP treated samples were also proceeded and no differences between the samples were noticed (Supplementary Figure S1). Samples were centrifuged for 10 min at 11,500 rpm at 4 °C, the supernatants were transferred to a 10 ml