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Organ-specific and genotype-dependent constitutive biosynthesis of secoiridoid glucosides in *Centaurium erythraea* Rafn, and its elicitation with methyl jasmonate

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ABSTRACT

While bioactive properties of *Centaurium erythraea* Rafn secoiridoid glucosides (SG) are widely recognized, many aspects related to their biochemistry, metabolism and relationship to the overall plant physiology are not yet understood. Here we present for the first time an insight into the molecular background of organ-specific and genotype-dependent constitutive biosynthesis of secoiridoids in *C. erythraea*, by comparing chemical profiles and secoiridoid glucosides-related gene expression. Genes encoding enzymes for intermediate steps of secoiridoids biosynthesis up to secologanin have been identified by analysing transcriptomic data from *C. erythraea* leaves. Results suggest an organ-specific capacity for the production and accumulation of secoiridoid glucosides, and highlight leaves as the main biosynthesis site. They also point out that significant differences in SG content among various *C. erythraea* genotypes, are, at least partially, determined by different expression patterns of SG-related genes. The biosynthesis of SG in *C. erythraea* leaves is enhanced upon treatments with methyl jasmonate (MeJA), which causes reprogramming of SG-related gene expression, leading to an increased production of valuable bioactive compounds. The present study unveiled several rate-limiting genes (encoding GES, G8O, 8HGO, IS and 7DLGT) in SG biosynthesis. *SLS* and *CPR* are highlighted as important genes/enzymes that might regulate biosynthetic flux through SG pathway. Information gathered within this study will help us gain deeper insight into the SG metabolism and develop strategies for enhanced biosynthesis of specific secoiridoid glucosides in homologous or heterologous systems.

1. Introduction

Centaurium erythraea Rafn, commonly known as centaury, is a biennial herbaceous plant that belongs to Gentianaceae family. It is widely distributed, inhabiting most of Europe, Caucasus, Iran and northern Africa (Chevallier, 2016). *C. erythraea* is known as an important source of pharmacologically valuable metabolites. Health promoting effects of centauries derive from the presence of many different types of specialized metabolites such as secoiridoid glucosides (SG) and phenolics (xanthones and flavonoids) (Šiler and Mišić, 2016). Various pharmacological effects attributed to centauries include stomachic, digestive, anti-inflammatory and anti-pyretic (Berkan et al., 1991; Newall et al., 1996), antimicrobial, choleretic, pancreatic and hepatoprotective activities (Kumarasamy et al., 2003; Šiler et al., 2010; Tuluce et al., 2011). Extracts of *C. erythraea* and other centauries have been used as additives and preservatives in food products and beverages, since they are considered as a rich source of natural antioxidants and compounds with antimicrobial properties (Božunović et al., 2018; Šiler et al., 2014). Main SG present in aerial parts of *C. erythraea* and other centauries are extremely bitter compounds sweroside (**3**), swertiamarin (**4**) and gentiopicrin (**5**) (Šiler et al., 2012; van der Sluis, 1985). These compounds exhibit fungitoxic, antibacterial (Šiler et al., 2010), gastroprotective (Niiho et al., 2006), hepatoprotective (Kondo et al., 1994), sedative (Bhattacharya et al., 1976), and antitumor (Ishiguro et al., 1988) activities.

Due to its pharmacological importance, *C. erythraea* has been

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intensively collected from its natural habitats. Overexploitation has led to a decrease in size and number of natural populations, which resulted in *C. erythraea* becoming an endangered species (Šiler et al., 2012). Numerous research efforts are being pursued with the aim to either find a sustainable pharmacological replacement for *C. erythraea* (Mišić et al., 2013), or to devise novel methods for more efficient production of SG in order to protect natural populations from disappearing and this species from extinction. An important prerequisite to achieve sustainable production of SG from alternative and renewable sources is the adequate comprehension of their metabolism, regulation and ecophysiological role in plants.

Biosynthesis of sweroside, swertiamarin and gentiopicrin, specialized metabolites characteristic for the family Gentianaceae, is presumed to go through secologanin (**2**), which is also a precursor for various monoterpenoid indole alkaloids (MIAs) (St-Pierre et al., 2013). MIAs are not found in family Gentianaceae, but are present in some other families of the order Gentianales. Very detailed studies on MIA biosynthesis, mainly in *Catharanthus roseus*, have been performed in the past decades. The main results of these investigations were cloning and identification of numerous biosynthetic and regulatory genes from MIA pathways. Most importantly, all the steps leading from geranyl diphosphate to the formation of secologanin (secoiridoid pathway) have been elucidated (Fig. 1) (for review, see Pan et al., 2016).

Biosynthetic capacity of plants for constitutive specialized metabolite production can be influenced by various factors. The production of the plant specialized metabolites is strongly dependent on the developmental stage, specific plant tissue or the plant genetic background. The expression level of the biosynthetic genes is often highly associated with the accumulation patterns of specialized metabolites. Some of the genes, though, could play a more important role than the others, and usually their levels of expression are strongly correlated with the levels of metabolites accumulation. These genes are usually referred to as "rate-limiting genes" because they exert regulatory roles in their biosynthetic pathways. From a biotechnological perspective, knowing which genes affect metabolite levels the most is crucial for metabolic engineering. We were interested in elucidating developmental characteristics, localization and genetic background of the biosynthesis and accumulation of secoiridoid glycosides in *C. erythraea*. The expression levels of the putative SG metabolic pathway genes were compared with total SG concentrations among SG high- and low-productive organs and genotypes of *C. erythraea*. Genes that could have a rate-determining function in the production of SG in *C. erythraea* have been identified here for the first time. Although SG are known to be produced constitutively, their inducibility with elicitors has been reported (Cao et al., 2016; Hua et al., 2014; Wang et al., 2010). To the best of our knowledge, in this work we have investigated for the first time a correlation between SG accumulation rates and their biosynthetic gene expression in MeJA elicited *C. erythraea* plants. The results point to the possibility of using elicitors as tools for the biotechnologically sustainable production of SG.

2. Results and discussion

2.1. Targeted UHPLC-MS/MS analysis of SG in C. erythraea methanol extracts

UHPLC-qqqMS quantitative analysis of *C. erythraea* methanol extracts was targeted towards one iridoid (loganin – **1**) and four secoiridoid (secologanin – **2**, sweroside – **3**, swertiamarin – **4** and gentiopicrin – **5**) compounds, which were unambiguously assigned, based on their retention times, MS, MS^2, and UV spectra, and comparisons with the reference standards. Molecular ions of targeted secoiridoids were visible as adducts of acetic acid which was used as a mobile phase, as previously described in Banjanac et al. (2017). Following PIS experiments and analysis of MS^2 spectra (Table S1), two diagnostic fragments of each compound were utilized in SRM experiment for the accurate quantification of targeted compounds (Fig. 2).

Iridoid glucoside loganin (**1**), which was eluted at Rt = 3.05 min, showed a pseudomolecular ion $[M + CH_3COOH - H]^-$ at *m/z* 449. Diagnostic MS^2 fragments of **1** were $[M - H - C_6H_{10}O_5]^-$ at *m/z* 227, resulting from the loss of glucose, and $[^{1,4}F]^-$ at *m/z* 127 (Sun et al., 2015). According to Sun et al. (2015), $^{1,4}F^-$ ion and [3-oxoMAQ–H]$^-$ ion are generated in MS^3 spectrum of **1**, due to the cleavage of pyran ring stemming from isomerization of hemiacetal group. Secologanin (**2**) with a pseudomolecular ion $[M + CH_3COOH - H]^-$ at *m/z* 447 was eluted at Rt = 3.72 min, and it yielded daughter ions $[M - C_6H_{10}O_5 - H_2O - CO - 24 - H]^-$ at *m/z* 155 via RDA cleavage, and $[M - C_6H_{10}O_5 - H_2O - CO - C_2O_2 - H]^-$ at *m/z* 123. A pseudomolecular ion $[M + CH_3COOH - H]^-$ at *m/z* 417 visible as a peak at Rt = 3.05 min was assigned to sweroside (**3**), and it generated product ion $[M - H - C_6H_{10}O_5]^-$ at *m/z* 195 by the loss of a glucose unit, and $[C_6H_{12}O_6 - H]^-$ at *m/z* 179, corresponding to deprotonated glucose.

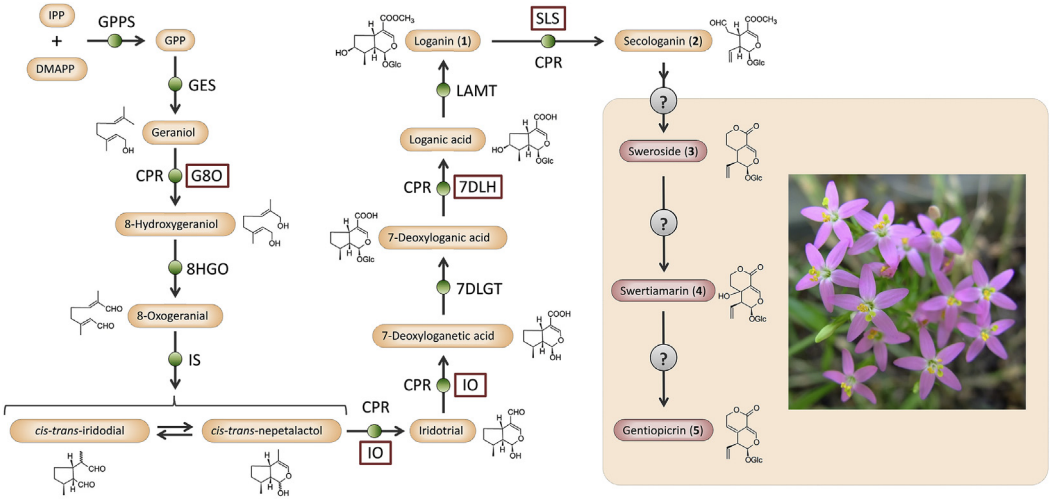

Fig. 1. The presumed biosynthetic pathway of secoiridoid glucosides in *C. erythraea*. GPPS – geranyl diphosphate synthase, GES – geraniol synthase, G8O – geraniol-8-oxidase, 8HGO-8-hydrohygeraniol oxidoreductase, IS – iridoid synthase, IO – iridoid oxidase, 7DLGT – 7-deoxyloganetic acid glucosyltransferase, 7DLH1 and 7DLH2 – 7-deoxyloganic acid hydrolase, LAMT – loganic acid O-methyltransferase, SLS – secologanin synthase. Cytochrome P450 enzymes, which require cytochrome P450 reductase (CPR) for their reactions, are indicated with red rectangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)