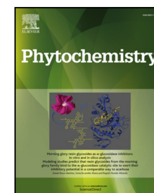
Phytochemistry 156 (2018) 142–150

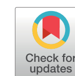
Contents lists available at ScienceDirect

Phytochemistry

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



Tissue-specific localization of polyketide synthase and other associated genes in the lichen, *Cladonia rangiferina,* using laser microdissection



Mostafa E. Elshobary[b], Michael G. Becker[a], Jenna L. Kalichuk[a], Ainsley C. Chan[a], Mark F. Belmonte[a], Michele D. Piercey-Normore[a,c,*]

[a] *Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, R3T 2N2, Canada*
[b] *Department of Botany, University of Tanta, Egypt*
[c] *School of Science and the Environment, Memorial University of Newfoundland (Grenfell Campus), Corner Brook, NL, A2H 5G4, Canada*

A R T I C L E I N F O

Keywords:
Cladonia rangiferina
Cladoniaceae
Algal gene expression
Apothecia
Atranorin
Fumarprotocetraric acid
Fungal gene expression
Laser microdissection
Thallus tissue

A B S T R A C T

The biosynthesis of two polyketides, atranorin and fumarprotocetraric acid, produced from a lichen-forming fungus, *Cladonia rangiferina* (L.) F. H. Wigg. was correlated with the expression of eight fungal genes (*CrPKS1*, *CrPKS3*, *CrPKS16*, Catalase (*CAT*), Sugar Transporter (*MFsug*), Dioxygenase (*YQE1*), C_2H_2 Transcription factor (C_2H_2), Transcription Factor PacC (*PacC*), which are thought to be involved in polyketide biosynthesis, and one algal gene, NAD-dependent deacetylase sirtuin 2 (AsNAD)), using laser microdissection (LMD). The differential gene expression levels within the thallus tissue layers demonstrate that the most active region for potential polyketide biosynthesis within the lichen is the outer apical region proximal to the photobiont but some expression also occurs in reproductive tissue. This is the first study using laser microdissection to explore gene expression of these nine genes and their location of expression; it provides a proof-of-concept for future experiments exploring tissue-specific gene expression within lichens; and it highlights the utility of LMD for use in lichen systems.

1. Introduction

Lichens are symbiotic associations composed of fungi (mycobionts) and their photosynthetic partners (photobionts), which may be either green algae or cyanobacteria. Mycobionts have evolved biosynthetic pathways that produce a plethora of specialized natural products rarely produced elsewhere (Oksanen, 2006; Stocker-Wörgötter, 2008). Most of these unique products, including types of depsides, depsidones, depsones, and dibenzofurans, are derived from the polyketide pathway (Wang et al., 2014b) or previously called the acetyl-polymalonyl pathway (Elix and Stocker-Wörgötter, 2008). Polyketide biosynthesis is catalyzed by a polyketide synthase (PKS), which assembles core polyketide molecules from carboxylic acid precursors and several malonyl-CoA units (Hopwood, 1997; Schümann and Hertweck, 2006). These specialized metabolites have potential pharmaceutical applications and antioxidant activities (Oksanen, 2006), elastase inhibitors (Proksa et al., 1994) and analgesics (de Sousa et al., 2002; Siqueira et al., 2010). In lichens, their functions include light screening (Solhaug and Gauslaa, 2012; Färber et al., 2014), snail antiherbivory (Asplund et al., 2010) and defense against viruses, microbes, protozoans, insects and fungi (Ingolfsdottir, 2002).

Polyketides are structurally diverse compounds that are synthesized by PKSs in the fungal symbiont of lichen associations. Despite growing knowledge of the diversity of PKSs and their polyketide products, the steps in the biosynthetic pathway are unclear. Fungal PKSs commonly consist of several well-defined domains, which are separated by short spacer regions. The domains include the ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains (Hopwood, 1997). The most notably conserved domain is the KS domain (Bingle et al., 1999), which may be useful for identification of PKSs in diverse organisms, for studying PKS evolution (Kroken et al., 2003), and to represent pathway associations (Ginolhac et al., 2005; Jenke-Kodama and Dittmann, 2009). The KS domain catalyzes condensation by polymerizing Acetyl-CoA to form a chain of eight carbons before cyclisation (Legaz et al., 2011). Additional domains underlie the structural diversity of PKSs which may also be further tailored by other genes and classified into different structural groups (reviewed in Hopwood and Sherman, 1990; Lal et al., 2000).

* Corresponding author. School of Science and the Environment, Memorial University of NL (Grenfell Campus), Corner Brook, NL, A2H 5G4, Canada.
E-mail addresses: mostafa_elshobary@science.tanta.edu.eg (M.E. Elshobary), umbeck26@myumanitoba.ca (M.G. Becker), ummilla8@myumanitoba.ca (J.L. Kalichuk), umchan62@myumanitoba.ca (A.C. Chan), mark.belmonte@umanitoba.ca (M.F. Belmonte), mpiercey-normore@grenfell.mun.ca (M.D. Piercey-Normore).

https://doi.org/10.1016/j.phytochem.2018.09.011
Received 22 April 2018; Received in revised form 25 August 2018; Accepted 28 September 2018
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Cladonia rangiferina (L.) F. H. Wigg., is a common lichen species in northern boreal regions (Shaver and Chapin, 1991), it is thought to be affected by climate change (Boudreault et al., 2015), and it has been used as a model species for investigating gene expression at early stages of lichenization (Athukorala and Piercey-Normore, 2014, 2015). *Cladonia rangiferina* produces two major compounds, atranorin (depside) and fumarprotocetraric acid (depsidone), which are thought to have properties related to light regulation (BeGora and Fahselt, 2001; Armaleo et al., 2008) and properties related to the habitat conditions (Culberson et al., 1977) or acidity tolerance (Hauck et al., 2009). The thallus tissue of *C. rangiferina* consists of an outer region with loosely arranged fungal hyphae enclosing algal cells and an inner region with highly compact fungal hyphae. The thallus grows acropetally with the younger, more photosynthetically active, portion near the top, which produces apothecia at the tips, and the older moribund portions near the base. If the function of atranorin and fumarprotocetraric acid is as stated above, transcriptional activity of *PKS* genes and other genes involved in their biosynthesis in the thallus tissue would be expected where their function is most effective – near algal cells or developing ascospores that require protection from harmful radiation.

Knowledge of where polyketides are produced in the thallus will improve our understanding of their ecological function, provide insights into the mechanisms by which they are regulated, and the environmental conditions which affect their synthesis. While these compounds are hypothesized to protect the algal symbiont and developing cells, few studies have localized their production. Scrobiculin production has been localized to asexual propagules, soredia (Asplund et al., 2010), and usnic acid to the thallus layer containing algae and within the sexually reproductive structures, apothecia (Culberson et al., 1993; Liao et al., 2010). Previous research also suggests that production may be triggered by supplementing the growth media with specific algal carbohydrates (Elshobary et al., 2016).

The goal of this study was to identify regions of lichen thallus with high levels of polyketide gene activity in *C. rangiferina* using laser microdissection (LMD) combined with targeted transcript profiling. Similar strategies have been used to monitor gene expression during plant fungal-interactions in root (Fiorilli et al., 2009), leaf (Becker et al., 2017), and stem (Abbott et al., 2010). This work identifies the location of PKS activity in the thallus of *C. rangiferina*, and it highlights the utility of LMD for use in lichen systems.

2. Results

The lichen thallus tissues used in this study and the location of the elements removed from each section by laser microdissection (LMD) are shown in Fig. 1. The cross section of the apical and basal portions of *C. rangiferina* thallus showed that the apical portion was smaller in diameter than the basal portion of the thallus (Fig. 1A and B). The inner fungal elements have been removed in Fig. 1D and E. The outer layer composed of loose fungal hyphae and clusters of algae was thicker in the apical portions of the thallus. However, the inner fungal layer was about the same thickness, but it was more intact in the apical than the basal portion of the thallus (Fig. 1A and B). The apothecium was located at the apex of the thallus stalk and it contained fungal tissue only (Fig. 1C and F). The entire apothecium has been removed in Fig. 1F.

The gene activity of nine genes that may be involved in polyketide biosynthesis was examined using real time qPCR. The results showed that the sugar transporter gene (*MFSsug2*) was upregulated in the apical portion more than in the basal portion, and the apical outer layer showed the highest relative gene expression more than the inner layer or apothecia (Fig. 2A). The two PKS genes tested (*CrPKS1* and *CrPKS3*) were upregulated in the apical outer layer more than any other layer (Fig. 2B and C). *CrPKS16* showed no significant difference in expression between the outer apical layer, both basal layers, and the whole apical region, but a lower expression level was shown in the apical inner layer and apothecia (Fig. 2D).

Dioxygenase (*YQE1*) was more highly expressed in the basal portion than in other parts of the thallus. The inner layer showed the highest *YQE1* transcription relative to the outer layer or apothecia. (Fig. 2E). Catalase (*CAT*) was the only gene in our study that was upregulated in both the outer apical and basal portions (Fig. 2F). The apothecium showed the lowest expression level for *CAT* (Fig. 2F). The C_2H_2 transcription factor was upregulated in both tissue layers of the apical thallus more than in the basal portion of the thallus (Fig. 2G). However, the *PacC* transcription factor showed a different pattern as it was upregulated in the basal inner portion more than the other tissues (Fig. 2H). The NAD-dependent deacetylase sirtuin 2 (*AsNAD*) gene of the photobiont showed the expected results where this gene was expressed only in the outer layer of both the apical and basal portions where the algal cells are located and expression was absent in the other tissues (Fig. 2I).

TLC results showed that the *C. rangiferina* thallus produced two distinct spots, a gray spot representing fumarprotocetraric acid (Rf = 0.2) and a yellow spot representing atranorin at (Rf = 0.8) (Fig. 3A). This is confirmed using HPLC which showed two distinctive peaks at Rt = 21.2 ± 0.5 min for fumarprotocetraric acid and at Rt = 23.7 ± 0.5 min for atranorin. The UV spectra of fumarprotocetraric acid and atranorin have three absorption maxima (Fig. 3B). Atranorin was observed to be present in the inner and outer thallus layers; however, fumarprotocetraric acid was observed to be present in the inner thallus layer and in the apothecia (Fig. 3A). The polyketide concentrations were determined using standard curves; fumarprotocetraric acid was 4.91295 μg/mg and atranorin was 14.9235 μg/mg.

The authenticity of atranorin and fumarprotocetraric acid in the acetone extract from the whole thallus of *Cladonia rangiferina* was confirmed by comparing the results of chromatogram and Mass Spectra of the extract with the spectra of the pure atranorin and fumarprotocetraric acid detected alone or in the mixture. The UPLC chromatogram of the acetone extract revealed two major peaks at retention times of 19.94 min and 23.75 min (Supplementary Fig.1 C), which were similar to those obtained for the pure metabolites (Supplementary Fig. 1 A, B): 19.73 min for fumarprotocetraric acid and 23.81 for atranorin. Furthermore, the fragmentation spectrum of both peaks was identical to the spectra of fumarprotocetraric acid and atranorin analyzed alone (Supplementary Fig. 1 C).

3. Discussion

This is the first study, to our knowledge, to use LMD to examine gene transcription in different tissues of a lichen thallus (*Cladonia rangiferina*). Three fungal PKS genes (*CrPKS1*, *CrPKS3* and *CrPKS16*), three other fungal genes (*MFSsug2, YQE1* and *CAT*) and two regulatory transcription factors (C_2H_2 and PacC) were hypothesized to be involved in polyketide biosynthesis according to Joneson et al. (2011). One green algal gene (*AsNAD*) was reported from early stages of thallus formation (Joneson et al., 2011). While different expression levels of these genes were shown in dissected thallus tissues, the apothecial sections showed low levels of expression for all genes. Apothecia were shown to produce usnic acid in other lichen species (Culberson et al., 1993; Liao et al., 2010) but the genes examined here may not be responsible for usnic acid (Abdel-Hameed et al., 2016).

3.1. Suggested roles of non-PKS genes

The upregulation of the sugar transporter (*MFSsug2*) gene in the outer apical layer compared with other layers corresponds to the area which is thought to be most active in sugar transport from the alga to the fungus (Adams et al., 2006). Once the sugar is transferred to the fungal partner it may be integrated into the polyketide production pathway. In this context, Wang et al. (2014a) demonstrated that sugars are transported through sugar transporters from the photosynthetic partner in *Endocarpon pusillum* Hedw. and converted into glucose or

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