# Symbiont-specific responses to environmental cues in a threesome lichen symbiosis

Jasmin Almer[1], Philipp Resl[2], Hörur Gudmundsson[3], Denis Warshan[3], Olafur Andresson[4], and Silke Werth[1]

[1]Ludwig-Maximilians-Universitat Munchen
[2]University of Graz
[3]University of Iceland
[4]Haskoli Islands

February 22, 2024

**Abstract**

Photosymbiodemes are a special case of lichen symbiosis where one lichenized fungus engages in symbiosis with two different photosynthetic partners, a cyanobacterium and a green alga, to develop two distinctly looking photomorphs. We investigated differential gene expression in photosymbiodemes of the lichen *Peltigera britannica* at different temperatures representing mild and putatively stressful conditions and compared gene expression of thallus sectors containing cyanobacterial photobionts with thallus sectors with both green algal and cyanobacterial photobionts. Firstly, because of known ecological differences between photomorphs, we investigated symbiont-specific responses in gene expression to temperature increases. Secondly, we quantified photobiont-mediated differences in fungal gene expression. High temperatures expectedly led to an upregulation of genes involved in heat shock responses in all organisms in whole transcriptome data. As expected, the expression of genes involved in photosynthesis was increased in both photobiont types at 15 and 25 °C. The green algae exhibited thermal stress responses mainly at 25 °C, the fungus and the cyanobacteria already at 15 °C, demonstrating symbiont-specific responses to environmental cues and symbiont-specific ecological optima. Furthermore, photobiont-mediated differences in fungal gene expression could be identified, with upregulation of distinct biological processes in the different morphs, showing that interaction with specific symbiosis partners profoundly impacts fungal gene expression.

## Symbiont-specific responses to environmental cues in a threesome lichen symbiosis

Jasmin Almer[1,2*], Philipp Resl[1,2], Hörur Gudmundsson[3], Denis Warshan[3], Ólafur S. Andrésson[3], Silke Werth[2*]

[1] Institute of Biology, University of Graz, Universitätsplatz 2, 8010 Graz, Austria

[2] Systematics, Biodiversity and Evolution of Plants, LMU Munich, Menzingerstraße 67, 80638 Munich, Germany

[3] Life and Environmental Sciences, University of Iceland, Sturlugata 6, 101 Reykjavik, Iceland

*Author for correspondence

**Key words:** differential gene expression, heat stress,*Peltigera britannica* (lichenized ascomycetes), photosymbiodemes, symbiotic interactions

**Abstract**

Photosymbiodemes are a special case of lichen symbiosis where one lichenized fungus engages in symbiosis with two different photosynthetic partners, a cyanobacterium and a green alga, to develop two distinctly looking photomorphs. We investigated differential gene expression in photosymbiodemes of the lichen *Peltigera britannica* at different temperatures representing mild and putatively stressful conditions and compared gene expression of thallus sectors containing cyanobacterial photobionts with thallus sectors with both green algal and cyanobacterial photobionts. Firstly, because of known ecological differences between photomorphs, we investigated symbiont-specific responses in gene expression to temperature increases. Secondly, we quantified photobiont-mediated differences in fungal gene expression. High temperatures expectedly led to an upregulation of genes involved in heat shock responses in all organisms in whole transcriptome data. As expected, the expression of genes involved in photosynthesis was increased in both photobiont types at 15 and 25 °C. The green algae exhibited thermal stress responses mainly at 25 °C, the fungus and the cyanobacteria already at 15 °C, demonstrating symbiont-specific responses to environmental cues and symbiont-specific ecological optima. Furthermore, photobiont-mediated differences in fungal gene expression could be identified, with upregulation of distinct biological processes in the different morphs, showing that interaction with specific symbiosis partners profoundly impacts fungal gene expression.

## 1 | Introduction

A lichen is a symbiotic organism composed of a fungal partner, the mycobiont, and a photosynthetic partner, the photobiont, which can be an alga and/or a cyanobacterium (Schwendener, 1868; Armaleo & Clerc, 1991). Lichens can be found in virtually every terrestrial ecosystem and due to their poikilohydric nature, they manage to survive even in harsh environments such as polar regions and coastal deserts and can withstand extreme temperatures as well as other abiotic stress factors (Werth, 2011). By association with locally adapted photobionts a lichenized fungus may be able to persist under diverse environmental conditions and to occupy large geographic ranges (Werth & Sork, 2014). For some lichen genera (e.g. *Peltigera* ), an association with a broad range of photobiont strains has been reported (Jüriado et al., 2019; Lu et al., 2018; O'Brien et al., 2005). Lichen-forming fungi are also able to switch photosynthetic partners, to form a stable symbiosis with a more compatible partner; incompatible symbiosis impedes thallus development and lichen growth (Beck et al., 2002; Insarova & Blagoveshchenskaya, 2016). The photobiont type can also determine the lichenized fungus's fitness by impacting its tolerance of ecological conditions (Ertz et al., 2018; Hyvärinen et al., 2002; Casano et al., 2011). A lichenized fungus's flexibility in choosing a suitable partner might therefore promote wide geographic distributions and it might also affect the establishment of the symbiosis within its environment (Ertz et al., 2018; Magain & Sérusiaux, 2014; Casano et al., 2011).

For several genera of lichenized fungi belonging to the Peltigerineae, including *Peltigera* , two distinct morphs have been described – chloromorphs containing green algae and cyanomorphs containing cyanobacteria as main photosynthetic partner; a form of lichen symbiosis referred to as a 'photosymbiodeme' (Green et al., 2002). These two morphs can grow as separate individuals but they may also grow as one single compound thallus with green algal and cyanobacterial sectors. Chloromorphs and cyanomorphs of lichens often show pronounced morphological and ecological differences (Hyvärinen et al., 2002; Green et al., 1993; Holtan-Hartwig, 1993), although they contain the same fungal species (Armaleo & Clerc, 1991). The type of photobiont can profoundly affect the ecology of the symbiotic association. For example, depending on its photobiont type, a lichen is able to tolerate stress to a greater or lesser extent; this has been shown for light stress (Demmig-Adams et al., 1990; del Hoyo et al., 2011) and oxidative stress (del Hoyo et al., 2011). Moreover, photobiont types can influence the photosynthetic performance (Green et al., 1993; Henskens et al., 2012), and enable the colonization of nutrient-poor habitats in the case of a cyanobacterial partner thanks to its nitrogen fixation (Goffinet & Hastings, 1994; Almendras et al., 2018; Hitch & Millbank, 1975). Thus, photobiont types can determine stress responses and ecology of lichens.

There are large differences between green algae and cyanobacteria with respect to physiology and cell morphology, which impact the way in which these photobionts can interact with their lichenized fungi. First of all, green algal photobionts are most often photosynthetically active at high ambient relative humidity (96.5%), while cyanobacterial photobionts require the lichen thallus to be hydrated by liquid water (Lange

et al., 1986). Secondly, green algal and cyanobacterial photobionts also differ markedly in the photosynthates which are transferred to the lichenized fungi, sugar alcohols like ribitol versus glucose (Richardson & Smith, 1968; Hill, 1972). Thirdly, they additionally differ in the structure and chemistry of their cell envelopes. The sturdy green algal cell walls contain cellulose (Domozych et al., 2012) and in the case of *Coccomyxa* the exceptionally resilient biopolymer sporopollenin (Honegger, 1984; Honegger & Brunner, 1981). In contrast, cyanobacterial cell envelopes are made of peptidoglycan encapsulated in a polysaccharide sheath (Hoiczyk & Hansel, 2000; Woitzik et al., 1988). Fourthly, although the formation of various chemotypes also depends on environmental factors (Cornejo et al., 2017; Culberson, 1986; Hale, 1957; Skult, 1997), photobiont type can affect the composition and content of carbon-based secondary compounds of lichens and chloromorphs have been reported to contain different secondary metabolites than cyanomorphs of the same fungal species (Kukwa et al., 2020; Tønsberg & Holtan-Hartwig, 1983). The different partners involved in the symbiosis can individually produce different secondary metabolites, and certain fungal metabolites are only produced in symbiosis with a specific photosynthetic partner. For instance, cyanobacteria – free-living and symbiotic ones – are able to produce toxins, e.g. when stressed (Kaasalainen et al., 2012; Gagunashvili & Andrésson, 2018). The effects of these toxins on the fungal and – in the case of tripartite lichens – the green algal partner as well as on other components of the lichen are still poorly known (Kaasalainen et al., 2009; Ivanov et al., 2021; Vančurová et al., 2018; Kaasalainen et al., 2012). Taken together, these marked physiological and structural differences imply that there must be different ways of interaction among partners, which should be reflected at the molecular level e.g., with respect to the fungal gene regulation depending on the interaction with specific symbiotic partners.

Stress responses are vital for survival and persistence of species in different environments, yet it is still not well understood under which conditions the different partners involved in lichen symbioses experience stress. In studies reporting gene expression of lichens exposed to temperature treatments, cyanobacterial photobionts expressed heat shock genes at lower temperatures than lichenized fungi (Steinhäuser et al., 2016), but green algal photobionts expressed heat shock at the same temperature as the lichenized fungus (Chavarria-Pizarro et al., 2021). Yet, to our knowledge, no study has so far addressed stress responses of cyanobacterial and algal photobionts simultaneously within the same compound lichen thallus. For symbiodemes, it is an important open question if the two photobiont types exhibit distinct stress responses at different temperatures.

Because they contain the same fungus and grow under the same environmental conditions, compound thalli with green algal and cyanobacterial sectors represent an ideal study system to explore photobiont-mediated differences in gene expression. The compound thalli can be exposed to identical experimental conditions as a closed system, which enables the examination of photobiont-mediated fungal gene expression and of photobiont-mediated gene expression of the symbiosis as a whole. Compound thalli are also ideally suited to address the question if the symbiotic partners share ecological optima. Previous observational field studies of lichen photosymbiodemes have shown morph-dependent habitat preferences (Elvebakk et al., 2008; Green et al., 1993; Holtan-Hartwig, 1993; Tønsberg & Holtan-Hartwig, 1983), which suggests that the symbiosis partners may differ in their ecological optima.

Here, we investigated differential gene expression in a photosymbiodeme-forming lichen that we exposed to different temperatures, including putatively stressful conditions, to test the hypothesis that the lichenized fungi and the green algal and cyanobacterial photobionts of compound thalli differ in ecological optima, causing them to experience thermal stress at different temperatures. Because of pronounced physiological and structural differences between green algal and cyanobacterial photobionts, we moreover hypothesized that the lichenized fungus exhibits differential gene expression mediated by the type of its photosynthetic partner. The results of this study are key to better understand how different partners influence the ecology of these enigmatic symbiotic organisms.

## 2 | Methods

### 2.1 | Study species

*Peltigera* is a cosmopolitan genus of lichen-forming fungi; its species form large foliose lichens which mainly

grow on soil and among bryophytes (O'Brien et al., 2009). Our study species *Peltigera britannica* (Gyelnik) Holtan-Hartwig & Tønsberg is a Holarctic species which prefers oceanic climates (Martínez et al., 2003). It forms a tripartite morph with the green alga *Coccomyxa* sp. and small amounts of the cyanobacterium *Nostoc* sp. embedded in granular structures on the upper side (cephalodia) and a bipartite morph containing only *Nostoc* sp. as photosynthetic partner (hereafter referred to as cyanomorph) (Pardo-De la Hoz et al., 2018; Holtan-Hartwig, 1993). The two morphs of *P. britannica* have a characteristic morphology (Fig. 1): when wet, the tripartite morph is apple green whereas the cyanomorph is of a bluish-gray color (Goffinet & Hastings, 1994). The photomorphs grow either as separate individuals or as a single compound thallus with cyanobacterial and tripartite sectors (Goward et al., 1995).

### 2.2 | Biological material

Four large compound thalli of *P. britannica* were collected at Heimörk nature reserve in southwestern Iceland, about 10 km southeast of Reykjavík in August 2017 (specimen 1 and 2: 64°4'5.04728"N, 21°43'38.87947"W; specimen 3: 64°4'5.19143"N, 21°43'39.02599"W; specimen 4: 64°4'5.44984"N, 21°43'40.89662"W). The lichen thalli were growing among mosses in shady and moist spots, usually in the opening of crevices of lava rocks or under birch shrubs. As compound thalli of*P. britannica* are not common in Iceland, they were only taken from sites where additional ones were present in order to prevent overharvesting.

### 2.3 | Experimental setup

After collecting the *P. britannica* specimens, they were exposed to a 12-hour light/12-hour dark cycle (13.3 µmol $m^{-2}s^{-1}$) in a cold room at 4 °C for five days in a closed Petri dish. At this temperature, they were first sampled (Fig. 2): pieces of thallus (ca. 1 $cm^2$ each) of the cyanomorph and the tripartite morph were cut off from all four specimens (treatment "4 °C_1"). The lichens were kept for 14 days at 4 °C and were watered regularly with deionized water to prevent desiccation. Once a week, Petri dishes were left open for the thalli to dry out to simulate the conditions in nature where they regularly dry out, and to avoid damages associated with permanent hydration. After this two-week period, the lichens were sampled again as before (treatment "4 °C_2"). Then, the fully hydrated lichens were transferred to a plant growth chamber (TOP-version KK-700 625-liter Climatic Chamber manufactured by POL-EKO-APARATURA; 13.1 µmol $m^{-2}$ $s^{-1}$) set to 15 °C, where they were left for two hours before additional samples were taken (treatment "15 °C"). In the last step, the lichens were exposed to 25 °C for two hours before sampling (treatment "25 °C"). At the higher temperatures, the specimens desiccated rapidly, therefore, they were sprayed with deionized water of the respective temperatures so they were fully hydrated during incubation and sampling. Immediately after harvest, samples were shock frozen in liquid nitrogen and stored at -80 °C until processing.

### 2.4 | Preparation steps, RNA extraction and RNA-sequencing

In order to successfully extract RNA, the samples were lyophilized at -58 °C and 31 µbar for 12 hours with a VirTis Sentry 2.0 (SP Scientific) to render the (green algal) cell walls brittle. Afterwards, the samples were again frozen in liquid nitrogen and pulverized with a bead mill (TissueLyser II, Qiagen). RNA was isolated with the innuPREP Plant RNA Kit (Analytik Jena) according to the manufacturer's instructions using the PL lysis buffer which resulted in the best RNA recovery in initial tests. RNA quality was assessed on a 2100 Bioanalyzer Instrument with the RNA 6000 Nano assay (Agilent). RNA libraries were constructed with dual indexing using the TruSeq® Stranded mRNA Library Prep kit (Illumina), which included a poly-A selection step to only sequence undamaged eukaryotic mRNA and exclude ribosomal RNAs. Initially, libraries with a mean fragment length of 300 bp were sequenced on a MiSeq platform (Illumina) with the MiSeq Reagent Kit v3 (2x75bp) at the University of Iceland in order to generate a reference transcriptome. Additionally, they were sequenced on the HiSeq 3000/4000 SR platform (Illumina), producing single-end reads of 50 bp at the Biomedical Sequencing Facility in Vienna which were used for gene expression analyses.

### 2.5 | Data analysis

To determine how the libraries needed to be normalized and pooled for HiSeq sequencing we used initial read counts of MiSeq data mapped to fungal and cyanobacterial reference genes (β-tubulin (AFJ45056.1) and

glycerol 3-phosphate dehydrogenase (AFJ45057.1) for fungus; protein translocase subunit *secA* (CP026681.1; region: 4141894 - 4142180) and RNase P RNA gene *rnpB* (CP001037.1; region: 1485004 - 1485242) for cyanobacteria). The quality of the MiSeq and HiSeq data was assessed using FastQC version 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/; accessed: 15.12.2019) and MultiQC version 1.1 (https://multiqc.info/; accessed: 25.02.2019). Poor quality base reads were removed with the FASTX-toolkit version 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/; accessed: 18.02.2019). Adapter sequences were trimmed with Trimmomatic version 0.36 (http://www.usadellab.org/cms/?page=trimmomatic; accessed: 20.02.2019). The processed paired-end MiSeq data was used for *de novo* transcriptome assembly with Trinity software version 2.4.0 (Haas et al., 2013). The quality of the assembly was assessed with the Trinity perl script TrinityStats.pl. The HiSeq data was pseudoaligned to the*de novo* transcriptome assembly with the RNA-seq quantification program kallisto version 0.45.0 (Bray et al., 2016). Coding regions were identified with TransDecoder (http://transdecoder.github.io; accessed: 20.03.2019). For the respective parameter settings see Electronic Supplementary Table S1.

### 2.6 | Differential gene expression analysis

Differential gene expression (DGE) analysis was conducted using the program DESeq2 version 1.22.2 (Love et al., 2014). The DESeq2 package has a normalization function implemented based on the median of ratios method, in which the geometric mean of the gene counts across all samples is used to calculate the ratios of each gene and each sample, allowing between-sample comparisons (hbctraining, DGE_workshop (2022), GitHub repository, [accessed 05.04.2022]; https://github.com/hbctraining/DGE_workshop). Additionally, a variance stabilizing transformation (VST) was performed on the data to remove variance-mean dependence (Anders & Huber, 2010). All genes were taxonomically assigned with MEGAN6 version 6.13.1 (Huson et al., 2007); only ascomycete, chlorophyte and cyanobacterial genes were retained for DGE analysis (after DGE analysis the term 'genes' will be used instead of 'transcripts' to be congruent with the terminology of 'differentially expressed genes'). The vst-normalized data of each of the three taxonomic units was used to perform Principal Component Analysis (PCA) (R version 3.5.2). In the DGE analyses, we quantified differences in fungal gene expression owing to morph type (tripartite vs. cyanobacterial) and those in fungal, algal and cyanobacterial gene expression owing to temperature. Transcripts with an adjusted Benjamini-Hochberg $p$ -value $< 0.05$ and a $\log_2$-fold change $> |2|$ were regarded as significantly differentially expressed. Our analyses focus on the 200 most significantly differentially expressed genes as determined with two-way ANOVAs for all organisms. Functional annotation of these differentially expressed genes was conducted using UniProt BLAST (The UniProt Consortium, 2021). The BLAST search was run using default settings with the target databases being "Fungi", "Plants" and "Bacteria". The best alignment based on e-value ($<10^{-5}$) was used to infer gene functions. The top-200 fungal differentially expressed transcripts were also blasted (blastx version 2.7.1+, translated nucleotide to protein) (Sayers et al., 2020) against our own database consisting of filtered metagenomic sequences of *Peltigera britannica* , *P. leucophlebia* and *P. collina* (unpublished data of the authors) using standalone BLAST for Linux Ubuntu (ncbi-blast+ package). This latter step was carried out to evaluate if the differentially expressed ascomycete genes were likely to originate from the lichen mycobiont or from other lichen-associated fungi. In the former case, there should be a hit both in the *P. britannica* metagenome, and in at least some of its congeners. The *P. britannica* metagenome was sequenced from a lichen individual not included in transcriptome sequencing (unpublished data by Werth, Andrésson, Resl and Warshan) and was built after *de novo* transcriptome assembly and DGE analysis. Gene Ontology (GO) annotations of all DEGs were conducted with the Bioconductor package *topGO* version 2.34.0 (Alexa & Rahnenführer, 2018).

## 3 | Results

### 3.1 |*De novo* transcriptome assembly with Trinity

Trinity assembly of the MiSeq data produced 274.600 transcripts including isoforms and all organisms, with an N50 of 1.240 (based on the longest isoform of each 'gene'). The N10 based on the longest isoform was 3.757 (Table S2).

### 3.2 | DESeq2 analysis

Overall, the number of differentially expressed transcripts per taxon was high, with 9,069 transcripts being assigned to ascomycetes, 25,109 transcripts to chlorophytes (green algae) and 2,476 transcripts to cyanobacteria (comprising only the longest isoform per 'gene'). A large number of genes was differentially expressed between tripartite morphs and cyanomorphs (hereafter referred to as photomorph-mediated), however, only ascomycete genes were considered for further analyses (312 genes differentially expressed; adjusted Benjamini-Hochberg $p$ -value $< 0.05$ and a $\log_2$-fold change $> |2|$). Additionally, many genes were differentially expressed when comparing 25 °C with the control temperature of 4 °C_1; in this case, genes from all three lichen symbionts were analyzed. At this temperature setting, 2,862 ascomycete genes, 9,275 green algal genes and 663 cyanobacterial genes were differentially expressed. DESeq2 analysis produced fewer differentially expressed genes when comparing 4 °C_1 with 15 °C (860 ascomycete, 3,258 algal and 148 cyanobacterial genes) as well as with 4 °C_2 (198 ascomycete, 4,095 algal and 53 cyanobacterial genes) (Fig. S1). Principal Component Analysis was used to assess the effects of temperature and photomorph on the overall expression pattern of the symbionts (Fig. 3, S2). On the one hand, the mycobiont (Fig. 3) clearly showed temperature-dependent expression, with clusters for low, medium and high temperatures. The photomorph-effect on mycobiont differential gene expression was less pronounced (2,862 temperature-mediated vs 312 photomorph-mediated differentially expressed ascomycete genes). On the other hand, the temperature-effect on green algal and cyanobacterial gene expression was low (Fig. S2).

### 3.3 | *Differentially expressed genes*

When comparing photomorphs, 123 of the 200 most significantly differentially expressed ascomycete genes were upregulated in the cyanomorph and 77 in the tripartite morph (Fig. 4A). Regarding gene expression at different temperatures, 103 of the 200 most significantly differentially expressed ascomycete genes were downregulated at 25 °C when compared to 4 °C_1, whereas only one cyanobacterial and one green algal gene were downregulated (Fig. 4B). We also checked the expression patterns of the 200 temperature-mediated DEGs at the other temperatures (Figs. S3, S4). Overall, of the top 200 DEGs, a somewhat higher proportion of photobiont genes than of ascomycete genes could be functionally annotated (cyanobacteria: 92.5%; green algae: 89%; ascomycetes: 81.5% (temperature-mediated) and 74% (photomorph-mediated)). Table 1 shows the top five significantly differentially expressed genes of each organism for the parameters in question (gene lists with the 200 top DEGs: Tables S3-S6).

#### 3.3.1 | *Ascomycete genes / photomorph*

GO annotations of the ascomycete DEGs illustrate a variety of distinct biological processes in the cyano- and the tripartite morph (Fig. 5). In both morphs, the majority of ascomycete DEGs were annotated to oxidation-reduction processes. In the tripartite morph, another substantial process was transmembrane transport, whereas the processes tricarboxylic acid cycle and phospholipid biosynthesis comprised a smaller number of DEGs. In the cyanomorph, the remaining ascomycete DEGs were annotated to carbohydrate metabolic processes and to protein phosphorylation. As these biological processes are relatively unspecific, the individual genes with the greatest significance were scrutinized with UniProt BLAST to obtain a more detailed picture of their putative functions.

About a quarter of the top 200 ascomycete DEGs could not be functionally annotated and 50.5% of the top 200 photomorph-mediated DEGs were also temperature-dependent (adjusted Benjamini-Hochberg $p$ -value $< 0.05$). In addition, the most significantly differentially expressed ascomycete genes were blasted to a local filtered metagenomic database we built using sequences of three species of *Peltigera*including *P. britannica* . Of the 200 most significantly differentially expressed ascomycete genes, only three could not be matched with our *Peltigera* database. These three genes were removed and substituted with the next three genes – which could be matched successfully – from the gene list (Tables S3-S6).

The highest level of differential expression of ascomycete genes was found for a transcript encoding an isopenicillin N synthetase which was expressed in the cyanomorph. The expression of this gene also showed a temperature response, being downregulated at 15°C. Other ascomycete genes upregulated in the cyanomorph

encoded cell wall synthesis proteins, e.g., SUN domain proteins and an alpha-1,3-glucan synthase; but proteins that seem to be responsible for cell wall synthesis were expressed in the tripartite morph as well, e.g., chitin synthase. Furthermore, there were indications of morph-dependent differential ascomycete gene expression regarding stress-responsive genes. For example, in the tripartite morph, a transcript encoding glutathione-S-transferase (GST) was upregulated, while in the cyanomorph, the upregulation of *para* -aminobenzoic acid synthase indicated stress response. We also found evidence of photobiont-mediated differential carbohydrate metabolism in the lichenized fungus. Various genes of carbohydrate pathways were found upregulated in either the cyanomorph (carbohydrate esterase family 4, α-1,2-mannosidase, various transporters) or the tripartite morph (galactonate dehydratase, D-xylose reductase). The results imply that processing of carbon compounds and provision of carbon differs among photomorphs.

#### 3.3.2 | *Ascomycete genes / temperature*

We found temperature-mediated differential expression of various ascomycete genes. Of the top 200 temperature-mediated ascomycete DEGs, 16% were photomorph-mediated as well. Many of the genes upregulated at 15 °C and 25 °C were stress-related, like genes encoding proteins directly responsible for heat stress responses such as heat shock proteins (HSP) and chaperonins (Fig. 6). On the other hand, some of the DEGs had an indirect role in stress responses. The latter included, among others, a small ubiquitin-related modifier (Rad60-SLD domain-containing protein) and ARPC5 (Actin-related protein 2/3 complex subunit 5). Furthermore, two hours of exposure to 25 °C led to the activation of transposons; in both morphs, various ascomycete genes encoding for proteins from transposon TNT 1-94 were upregulated as well as one gene that was identified as a retrotransposable element.

In addition to upregulation of genes involved in stress responses, downregulation of a large number of genes was observed at 25 °C. These genes could often only be annotated roughly, e.g., to enzyme classes like oxidases and hydrolases or transporter proteins like those of the major facilitator superfamily. Genes that could be annotated more thoroughly were part of various pathways, including translation and transcription as well as some genes encoding mitochondrial proteins. GTPase activity and GTP-binding, ATPase activity as well as NAD(P)-binding were major functions downregulated at 25 °C.

#### 3.3.3 | *Cyanobacterial genes / temperature*

Functional annotation of cyanobacterial genes exposed to the temperature treatments revealed a number of upregulated genes that had two main functions: stress responses and photosynthesis. The former comprises a group of genes encoding HSPs and chaperonins as well as other genes involved in stress response mechanisms, including modulators (e.g. Dps, lysine–tRNA ligase, Bax inhibitor-1), various response regulators of signaling cascades, genes involved in DNA repair (e.g. *recA* ) and an antibiotic (bleomycin) resistance protein; these were upregulated at 15 °C and 25 °C (Fig. 6). Photosynthesis genes, which were upregulated at 15 and 25 °C, performed various photosynthetic functions of both the photosystem I and II as well as the cytochrome complex and the ATP synthase.

#### 3.3.4 | *Green algal genes / temperature*

The temperature-stress induced DGE results for the green algae were similar to those of the cyanobacteria. Most of the green algal genes upregulated at increased temperatures could be functionally annotated to genes encoding various photosynthetic proteins and to stress-response proteins. Genes encoding stress-response proteins, including HSPs, chaperonins as well as proteins for DNA repair mechanisms and signal transduction were upregulated mainly at 25 °C (Fig. 6). Furthermore, at 25 °C, increased expression of proteins associated with lipid metabolism (e.g., sterol 14 desaturase, 3-hydroxy-3-methylglutaryl coenzyme A synthase, prolycopene isomerase) was observed. The main biological process attributed to lipid metabolism by topGO analysis was “lipid metabolic process”; other lipid-metabolism related biological processes included lipid transport and carotenoid biosynthetic process.

## 4 | Discussion

This study reveals functional characteristics of lichen symbioses by contrasting gene expression patterns

of the fungal partner growing in associations with different photobionts, i.e., thallus sectors containing cyanobacteria or green algae as predominant photosynthetic partners. We focused specifically on the fungal genes differentially expressed between morphs as well as three different temperature treatments, and on temperature-related differential expression of green algal and cyanobacterial genes. Our analyses reveal fungal gene expression differences mediated by different photobionts and temperatures. In the threesome partnership of *P. britannica* , stress responses are triggered at markedly different temperatures in cyanobacteria, lichen-forming fungi and green algal symbionts.

### 4.1 | *Ascomycete genes / photomorph*

Differential gene expression in lichen-forming fungi is mediated by interactions with different photosynthetic partners. For example, the upregulation of an ascomycetous isopenicillin N synthetase in the cyanomorph could be attributable to mycobiont-photobiont interactions. Metabolic interactions between lichenized fungi and their photosynthetic partners have been shown to be able to affect the production of lichen substances (Shrestha & St. Clair, 2013), some of which have antibiotic (Gazzano et al., 2013; Shrestha & St. Clair, 2013) or growth-inhibitory properties (Ocampo-Friedmann & Friedmann, 1993; Ranković & Mišić, 2008). Penicillin is a known fungal β-lactam antibiotic which mainly controls gram positive bacteria (Holtman, 1947) and isopenicillin N synthetase is essential for the production of penicillin (Müller et al., 1991). By producing this antibiotic, the lichenized fungus in our study might be able to control growth and population size of the gram-positive part of its bacterial microbiome and potentially also of its intrathalline cyanobionts. This is further indicated by the upregulation of fungal genes encoding velvet domain-containing proteins in the cyanomorph, as these genes also play a role in secondary metabolism and antibiotic biosynthesis in model fungi *Penicillium* and*Aspergillus* (Kopke et al., 2013; Kato et al., 2003). Excessive growth of *Nostoc* photobionts may be detrimental to the symbiosis, as *Nostoc* produces toxins in various lichen species, especially in those growing in humid climates (Kaasalainen et al., 2012), such as*P. britannica* . The mycobiont could be able to avoid cyanotoxin-induced damage by expressing isopenicillin N synthetase and velvet domain proteins in the cyanomorph. Subsequent studies are necessary to test this hypothesis. Currently it remains unclear if other*Peltigera* species living in symbiosis with only cyanobacterial photobionts also express genes involved in the production of antibiotics. We hypothesize that the regulation of cyanobacterial growth could be important for maintaining long-term cohesion of large*Peltigera* thalli under varying environmental conditions. The upregulation of the antibiotic proteins depended on temperature as well, but the pattern was not uniform. This suggests upregulation results from a combination of factors, like the presence of *Nostoc* and temperature changes.

In either photomorph, different genes responsible for cell wall synthesis or cell wall modification were upregulated, e.g. SUN domain proteins in the cyanomorph and chitin synthase in the tripartite morph (downregulated at 25 °C) (Gastebois et al., 2013; Garcia-Rubio et al., 2020; Bowman & Free, 2006). Fungal hyphal structures and their cell wall play a vital part in the infection of host organisms such as plants (Hopke et al., 2018) and in the process of host interaction (Geoghegan et al., 2017) – as is the case in lichen symbioses (Kono et al., 2020; Honegger, 1986). In addition, various environmental factors influence the remodeling of fungal cell walls (Patel & Free, 2019). The lichenized fungus might interact differently with its two photosynthetic partners and, mediated by its respective partner, different aspects of cell wall (trans)formation may be required to establish contact sites. The green algal partner *Coccomyxa* contains resilient sporopollenin biopolymers in its cell walls, which the fungus cannot penetrate or degrade (Honegger & Brunner, 1981). Instead of haustoria, the mycobiont forms wall-to-wall appositions (Honegger, 1984). The cyanobacterial partner *Nostoc* usually isn't penetrated either (Honegger, 1984, 1985), but the hyphae encircle the *Nostoc* cells tightly and sometimes even invaginate them (Pawlowski & Bergman, 2007). Also, in *Peltigera* species with *Nostoc* , intrawall haustoria have been described, which penetrate the membrane of the cyanobacterial cell wall (Koriem & Ahmadjian, 1986). The establishment of these contact sites has an effect on the symbiotic relationship as they allow nutrient transfer between the symbionts (Kono et al., 2020) which is often considered the functional core of lichen symbioses. However, differential gene expression of cell wall modifying genes induced by photosynthetic partners may impact lichens on a much broader scale. They could be involved in the formation of the strikingly different phenotypes which *P. britannica* (alongside other lichens) develops

with the goal to optimize different aspects of the symbiotic relationship. In foliose lichens (such as *Peltigera* ) the mycobiont actively positions the photobionts within the thallus to ensure optimal acclimatization to its environment (Honegger, 2012). The mycobiont might also control growth and proliferation of the photobionts (Honegger, 2012; Hyvärinen et al., 2002). However, co-development of the symbionts is needed for the successful establishment of a lichen thallus (Hill, 1985). Conclusively, the differential expression of cell wall (trans)formation genes between morphs could cause the different phenotypes or at least contribute to their formation.

The interaction between mycobiont and photobiont can also be seen in the upregulation of ascomycete stress response genes, e.g., that of glutathione-S-transferase (GST) in the tripartite morph and that of*para* -aminobenzoic acid (PABA) synthase in the cyanomorph; the expression of both of these enzymes was temperature-dependent as well, as they were downregulated at 25 °C. GSTs are, inter alia, active during oxidative stress (Morel et al., 2009). As desiccation leads to an accumulation of reactive oxygen species (ROS), oxidative stress occurs in desiccating organisms, including lichens (Kranner et al., 2008; Holzinger & Karsten, 2013). A major player providing tolerance towards oxidative stress is glutathione (GSH), an antioxidant which reduces ROS, whereby GSH itself is being oxidized into glutathione disulfide (GSSG) (Kranner et al., 2008). The enzyme GST is a major driver of this detoxification process as it is responsible for the conjugation of GSH onto ROS (Kammerscheit et al., 2019). Lichens that tolerate desiccation to a higher degree usually contain an increased GSH pool in their hydrated state, enabling a rapid oxidation to GSSG during desiccation and causing an increased GSSG pool (Kranner, 2002). The upregulation of GST in our samples could therefore be a result of initial desiccation and desiccation-induced accumulation of ROS, even though the thalli were watered regularly and were sampled while fully hydrated. The upregulation of GST occurred in the tripartite morph only – although both morphs received identical treatment. This pattern might be due to an increased desiccation tolerance of lichens with green algal photobionts, relative to those with cyanobacteria (Kranner, 2002), or it might reflect inherent differences in physiological properties among photomorphs, e.g. water holding capacities of the gelatinous sheath of*Nostoc* sp. (Liang et al., 2014). Future studies should conduct specific experiments to characterize these putative physiological differences. Furthermore, the expression of PABA synthase in the cyanomorph indicated stress response, as this enzyme has been shown to improve tolerance to thermal stress in *Agaricus bisporus* (Lu et al., 2014) and to enhance UV-C resistance in *Arabidopsis thaliana*(Hu et al., 2019). As cyanobacteria are more susceptible to heat stress (see below) as well as high light when hydrated than green algae (Gauslaa et al., 2012), the expression of PABA synthase could be a response of the lichenized fungus to environmental stress in which the mycobiont supports the photobiont in keeping the holobiont vital.

Photomorph-dependent differential expression of genes involved in fungal carbon metabolism were expected as both partners produce distinctive carbon-based secondary compounds. Green algae like *Coccomyxa*produce polyols such as ribitol (Richardson & Smith, 1968), whereas cyanobionts produce glucans and glucose (Hill, 1972), all of which are translocated and taken up by the mycobiont. In the mycobiont, these carbohydrates are converted into the energy-storing compound mannitol (Palmqvist et al., 2008; Grzesiak et al., 2021). The obtained sugars either serve a nutritional purpose (i.e. growth and respiration) (Palmqvist et al., 2008; Smith, 1963) or they are conducive to stress tolerance (e.g. protection during desiccation) (Farrar, 1976; Spribille et al., 2022). As the carbon-based substrates are distinct, the mycobiont requires different enzymes for substrate transport and transformation. Although various genes responsible for carbohydrate metabolism were found upregulated in both morphs, none of these could be assigned directly to glucan or ribitol metabolism. Yet, a number of studies have shown the complex nature of carbohydrate movement within lichens and have emphasized photobiont-induced distinctions (Smith et al., 1969; Hill, 1972; Richardson et al., 1967; Palmqvist et al., 2008; Hill & Ahmadjian, 1972; Kono et al., 2020). In our study, the expression of ascomycete genes involved in carbon metabolism was temperature-dependent in some cases, and some genes (e.g., carbohydrate esterase family 4 and galactonate dehydratase) were upregulated at 4 °C_1 and 4 °C_2, which might have to do with cold tolerance mechanisms, as carbon metabolism plays a role in tolerance and acclimation to cold. In photosynthetic organisms, primary as well as secondary carbon metabolites have been proven to be essential to withstand cold temperatures (Fürtauer et al., 2019; Calzadilla et al., 2019; Tarkowski & Van

den Ende, 2015). The lichenized fungus might respond similarly to cold temperatures by metabolizing the carbohydrates it obtains from its photosynthetic partners; indeed, previous studies have shown that polyols, such as ribitol and mannitol, serve as cryoprotectants (Fontaniella et al., 2000; Hájek et al., 2009).

We found over 300 photomorph-mediated differentially expressed genes in the lichenized fungus. More than half of these genes were also differentially expressed at different temperatures. Therefore, the differential expression of these ascomycete genes appears to be the result of a combination of factors – photobiont type plus specific stimulus. However, the results clearly indicate photobiont-mediated differential fungal gene expression.

4.2 | *Ascomycete genes / temperature*

A stepwise temperature increase from 4 to 25 °C resulted in the upregulation of various stress-response genes in the lichenized fungus, with upregulation beginning already at 15 °C. Heat shock proteins and chaperonins are proteins directly involved in stress responses, giving a clear indication that the organism is stressed at elevated temperatures when it has been pre-acclimated to cold; as does the upregulation of proteins which are only indirectly involved in stress response mechanisms, such as the Rad60-SLD domain and ARPC5. SLDs are SUMO-like domains and – as SUMO (small ubiquitin-like modifier) proteins – SLDs are responsible for the SUMOylation of a range of other proteins (Ghimire et al., 2020; Prudden et al., 2009). Protein SUMOylation is of great relevance as it renders targeted proteins useful for various vital biological processes. Heat stress has been described as one of the factors leading to increased SUMOylation activity (Zhou et al., 2004). Shortly after a rise in temperature, SUMO conjugates accumulate, indicating that SUMOylation might be an early stress response system (Kurepa et al., 2003). SUMOylation is the starting point of a cascade of cellular processes in reaction to stress as it activates target proteins, such as heat shock factors, which in turn activate specific proteins, such as heat shock proteins (Kurepa et al., 2003). Activation of HSPs as a consequence of SUMOylation has been described for various organisms, including *A. thaliana* (Kurepa et al., 2003) and *Candida albicans* (Leach et al., 2011). The upregulation of Rad60-SLD in *P. britannica* photomorphs at 15 and 25 °C therefore indicates fungal responses to thermal stress. Similarly, the expression of ARPC5 at 25 °C reflects stress response processes. ARPC5 is a member of the multiprotein complex Arp2/3; in the nucleus, the Arp2/3 complex contributes to DNA repair mechanisms as it promotes migration of DNA double-strand breaks which are to be repaired (Schrank et al., 2018). An upregulation of DNA repair mechanisms at high temperatures is expected because elevated temperatures can cause heat-induced DNA damage (Oei et al., 2015; Steinhäuser et al., 2016).

Furthermore, in both photomorphs, an upregulation of ascomycete transposon genes was detected at 25 °C. Transposons, or transposable elements (TE), are DNA sequences which can change their position in the genome (Muñoz-López & García-Pérez, 2010). Transposon translocations can affect gene functioning, especially when they are inserted into a gene's coding region. The movements of TEs are subject to prior activation; stress conditions can serve as stimuli for TE activation (Dubin et al., 2018). Increased TE transcription has been described for other organisms experiencing heat stress, such as *A. thaliana* (Huang et al., 2018). In the pathogenic ascomycete fungus *Magnaporthe grisea,* heat stress, copper sulfate and oxidative stress cause activation of retrotransposons (Ikeda et al., 2001). Therefore, the upregulation of TEs in our *P. britannica* specimens at 25 °C might result from thermal stress. The biological consequences of stress-related TE translocations (Negi et al., 2016) would be an interesting area of future studies.

In addition to upregulation of ascomycete stress response genes, downregulation of a large number of genes was observed at 25 °C. Functional annotation of these genes proved difficult, as many could only be annotated roughly (e.g., to enzyme classes). Genes that could be annotated more precisely were mostly part of regular metabolic pathways. GTPase and ATPase activity as well as NAD(P)-binding were major functions downregulated at 25 °C. The same is true for various genes responsible for translation and transcription and for some genes encoding mitochondrial proteins. The latter suggests a reduction of mitochondrial function; similar results have been described for stressed *Saccharomyces cerevisiae* cells (Sakaki et al., 2003). Curbed metabolism in stress situations might be beneficial to allocate the available energy resources to stress response pathways, allowing organisms to survive under suboptimal conditions (Peredo & Cardon, 2020).

The results illustrate that an organism exposed to heat stress does not solely react by means of expression of stress genes but also by downregulation of other genes such as those involved in metabolic pathways under normal conditions. In the lichenized fungus, heat stress responses such as upregulation of HSPs were already induced at 15 °C; at 25 °C, the fungus appeared to be highly stressed. Therefore, long-term exposure to high temperatures could result in severe damage, especially when the lichen's respiration rate increases, causing a negative carbon balance over extended time periods (Sundberg et al., 1999; Lange & Green, 2005).

Interestingly, of the 200 ascomycete genes most significantly differentially expressed between temperatures, 103 were downregulated at 25 °C, and 27 of these downregulated genes were also differentially expressed between photomorphs. Of the 97 fungal genes upregulated at 25 °C, only seven were photomorph-mediated. Stress-related proteins are highly conserved (Elliott, 1998) and stress responses are vital for survival, which could explain why their upregulation at 25 °C occurs largely independent of associations with a specific partner.

4.3 | *Cyanobacterial genes / temperature*

The relatively low number of just below 2,500 cyanobacterial transcripts detected in our samples results from the method of library construction involving selection of poly-A mRNA. Cyanobacterial transcripts were found nonetheless due to carry-over, but as a result the number of cyanobacterial genes with significantly different expression was relatively small. Provided that this carry-over is a random process, cyanobacterial transcripts would be sampled depending on their frequency in the RNA pool; nevertheless, the results have to be interpreted with due caution.

Increased temperature led to significant differential expression of (heat) stress and photosynthesis-related cyanobacterial genes. The former category comprises HSPs, chaperonins and Dps (DNA-binding protein from starved cells), as well as genes indirectly involved in stress response mechanisms, e.g. lysine-tRNA ligase. Dps is a highly conserved protein which is part of various stress response pathways (Karas et al., 2015). Its two main functions in stress responses are DNA binding – i.e. shielding the DNA from reactive chemicals – and ferroxidase activity. Ferroxidase oxidizes $Fe^{2+}$ to $Fe^{3+}$, and thereby prevents the formation of hydroxyl radicals via the Fenton reaction ($Fe^{2+} + H_2O_2{}^{3+} + OH^- + {}^*OH$) (Calhoun & Kwon, 2011; Nair & Finkel, 2004). The lysine-tRNA ligase is responsible for the formation of lysyl-tRNA which is of relevance in protein synthesis, transferring lysine into ribosomes (Wu et al., 2007). Besides, the enzyme has another function – as has been assessed for*Escherichia coli* – as it synthesizes various adenyl dinucleotides, particularly $Ap_4A$. This function of lysine-tRNA ligase is active only under stress and leads to an accumulation of $Ap_4A$. $Ap_4A$ serves as a modulator of heat shock response, binding to and modifying stress proteins (Onesti et al., 1995).

The second category of genes upregulated at higher temperatures in our samples are photosynthetic genes. These genes were annotated to various photosynthetic functions of photosystems I and II as well as the cytochrome complex and ATP synthase. Expectedly, higher temperatures led to an increase in photosynthetic activity (Lommen et al., 1971), but the expression of photosynthetic genes might as well be caused by heat-induced structural changes of the photosynthetic machinery, such as protein complexes (Allakhverdiev et al., 2012; Ivanov et al., 2017).

A cyanobacterial gene encoding a bleomycin resistance protein was highly significantly upregulated at 15 degC and 25 degC, but the reason for its upregulation is difficult to determine. This protein confers resistance to the antibiotic bleomycin (Dumas et al., 1994). In *E. coli* it has been shown that the presence of antibiotic resistance genes likely has been induced by adaptation to stress (like thermal stress) as tolerance towards antibiotics underlies similar mechanisms as tolerance towards heat (Cruz-Loya et al., 2019) This might potentially explain why a gene encoding a bleomycin resistance protein was upregulated at elevated temperatures in the *Nostoc* cyanobiont of *P. britannica* . Besides, Keszenman et al. (2000 & 2005) have demonstrated that the upregulation of bleomycin resistance genes is a side effect of heat stress in *S. cerevisiae* , as the yeast cells proved to be resistant to bleomycin treatment after having been exposed to heat stress. This correlation between heat stress and bleomycin resistance is probably the result of cross-linking of DNA repair mechanisms (Keszenman et al., 2000, 2005). A potential role of bleomycin-resistance genes in DNA

repair has already been proposed in previous studies on *E. coli* (Blot et al., 1991).

A range of cyanobacterial genes of the *P. britannica*photosymbiodemes show temperature-mediated differential gene expression. Most DEGs are responsible for either heat stress responses or photosynthesis. In general, our results demonstrate that in cyanobacteria, heat stress is induced at a temperature of 15 degC already, similar to what we found in the lichen-forming fungus. This coincides with personal observations that *P. britannica* cyanomorphs and compound thalli only grow in shady and moist habitats where environmental fluctuations are minimal. The preference of these sites might simply reflect the cyanobionts' limited tolerance towards high temperatures and desiccation. The upregulation of genes of the photosynthetic apparatus at higher temperatures suggests an increase in the cyanobacteria's photosynthetic activity. Hence, elevated temperatures are not merely a stressor but are beneficial for cyanobacteria to a certain extent. It seems, however, unlikely that the favorable conditions for photosynthesis counterbalance the detrimental effects caused by heat stress, otherwise the cyanomorphs and compound thalli would not be habitat-restricted to the coolest, moistest sites available.

4.4 | *Green algal genes / temperature*

Similar to the cyanobacterial genes, most of the green algal genes upregulated at higher temperatures were either part of photosynthetic or stress response pathways. HSPs, chaperonins as well as proteins for DNA repair and signal transduction were expressed primarily at 25 degC. This suggests that the green algal partner tolerates heat to a greater extent than the cyanobacterial and fungal partners. This result is consistent with findings of Green et al. (2002) as well as personal observations that *P. britannica* tripartite morphs grow in relatively open habitats exposed to more fluctuations in temperature, light and water availability. Furthermore, photosynthetic activity of the algae was enhanced at 15 degC and 25 degC, so tripartite morphs may partly benefit from a rise in temperature. However, the actual optimal photosynthetic temperature is difficult to determine, as it is species-dependent (Wagner et al., 2014) as well as dependent on various other environmental factors (Alam et al., 2015; Green et al., 2002). Given the upregulation of photosynthetic genes at 15 and 25 degC, one can conclude that both temperatures are within the range of optimal temperature for net photosynthesis in *P. britannica* photosymbiodemes. However, as 25 degC leads to an expression of genes relevant for heat shock responses, a long-term exposure to higher temperatures could – at least partly – inactivate the photosynthetic apparatus (Ivanov et al., 2017). A prolonged increase in temperature could also negatively impact carbon balance if the lichen's respiration rate outweighs its photobiont's photosynthesis rate. Elevated respiration after temperature increases has been described for lichens and their photobionts (Palmqvist et al., 2008; Sundberg et al., 1999), however, the respiration rate usually normalized after one to three hours (Sundberg et al., 1999). There is no evidence in our dataset that a rise in temperature led to an elevated algal respiration rate; based on this the carbon balance in the lichen is most likely still positive. Gas-exchange measurements would be useful to settle this issue, but were beyond the scope of the current study.

We also observed upregulation of proteins associated with lipid metabolism at 25 degC. This metabolic activity could result from lipid remodeling induced by heat stress, especially in regard to membrane lipids. Heat can compromise the structural integrity of membranes and consequently an organism's vitality. In order to counteract membrane disintegration, a variety of lipids are synthesized and accumulated in the cell (Zhang et al., 2020), such as saturated fatty acids (Barati et al., 2019), whereas other lipids, mostly polyunsaturated fatty acids, undergo selective degradation or are converted to storage lipids (Legeret et al., 2016). These metabolic conversions of lipids seem to allow the algae to cope with an increase in temperature (Zhang et al., 2020; Song et al., 2018). Therefore, the expression of lipid metabolism proteins at 25 degC might be an indirect response to stress induced by elevated temperatures.

These results help us in understanding the ecological conditions under which lichen symbioses grow in nature. Compound thalli of photosymbiodemes represent an attractive study system as both photomorphs grow under the same environmental conditions, so both photobionts do not only have to tolerate these conditions but must also benefit from them to establish a successful symbiosis. Therefore, photosymbiodemes are restricted in their distribution to certain ecological niches (Green et al., 1993; Lange et al., 1988; Purvis, 2000), a

circumstance we were also able to observe in our study, as the*P. britannica* specimens only grew in damp, relatively hidden spots such as small crevices of lava rocks or under branches of birch shrubs growing on a small slope. Solitary tripartite morphs, on the other hand, grew in open areas that are more exposed to environmental fluctuations, especially regarding light, temperature and humidity. Solitary cyanobacterial thalli were missing in the collection area at Heidmork. These distribution patterns of *P. britannica* seem to be indicative of the respective photobiont's stress tolerance. More studies of gene expression in lichen photomorphs are needed to understand to which extent the patterns reported here hold true for other taxa as well.

## Conclusions

This is the first study reporting differential gene expression of lichen photomorphs based on compound thalli. Our differential gene expression analyses illustrate the effects of temperature stress on all partners involved in the lichen symbiosis and show a distinct photomorph-mediated fungal gene expression pattern in *P. britannica* influencing mycobiont-photobiont contact and management of photobiont growth. This suggests an interplay and plasticity of fungal, cyanobacterial and algal gene expression in lichen symbioses that was previously undocumented. Moreover, our results show that the symbiosis partners possess different temperature optima. Each organism in the lichen symbiosis, be it the fungus, the alga, or the cyanobacterium, reacted to thermal stress – and each organism did so in a distinct manner. The fungi and cyanobacteria were heat stressed at 15 degC already, whereas the green algae were heat stressed only at 25 degC. Our study offers novel insights into how symbiotic partners in *P. britannica* photomorphs manage their interactions and responses to environmental factors such as temperature through differential gene expression. We hope that our results contribute to a better understanding of how different photosynthetic partners can influence the ecology of lichens.

## Acknowledgments

This study was funded by the Icelandic Research Fund IRF (Grant # 141102-051 and # 174307-051). SW was funded by a Marie Curie IEF scholarship ('Lichenomics') under the FP7 Framework Program of the European Commission. The lab of Silke Werth was supported by Startup funding from LMU Munich. Export permits for biological material were obtained from the Icelandic Institute of Natural History (permit # 2015090014/43-0 and # 2017010019/43-0). We thank the Biomedical Sequencing Facility in Vienna for sequencing the HiSeq libraries.

## Author contributions

JA wrote the first draft of the manuscript, did field work, the experiments, wet lab work and data analyses. PR contributed substantially to data analyses. HG performed RNA-seq library preparation with JA. DW did preliminary analyses of MiSeq transcriptome data that were used to decide on pooling for HiSeq. OSA oversaw the lab work at University of Iceland. SW conceived and designed the study, did field work, helped with the temperature shift experiment, and contributed to data analyses and manuscript writing.

## Data availability statement

The data is available upon request from JA.

## References

Alam, M. A., Gauslaa, Y., & Solhaug, K. A. (2015). Soluble carbohydrates and relative growth rates in chloro-, cyano- and cephalolichens: Effects of temperature and nocturnal hydration.*New Phytologist, 208* (3), 750-762. doi:https://doi.org/10.1111/nph.13484

Alexa, A., & Rahnenfuhrer, J. (2018). topGO: Enrichment Analysis for Gene Ontology. R package version 2.34.0.

Allakhverdiev, S., Kreslavskii, V., Fomina, I., Los, D., Klimov, V., Mimuro, M., Mohanty, P. & Carpentier, R. (2012). Inactivation and repair of photosynthetic machinery under heat stress. In S. Itoh, P. Mohanty,

& K. N. Guruprasad (Eds.), *Photosynthesis: Overviews on Recent Progress and Future Perspective* (pp. 187-214). New Delhi: International Publishing House Pvt. Ltd.

Almendras, K., Garcia, J., Caru, M., & Orlando, J. (2018). Nitrogen-fixing bacteria associated with *Peltigera* cyanolichens and *Cladonia* chlorolichens. *Molecules, 23* (12), 3077. doi:10.3390/molecules23123077

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biology, 11* (10), R106. doi:10.1186/gb-2010-11-10-r106

Armaleo, D., & Clerc, P. (1991). Lichen chimeras: DNA analysis suggests that one fungus forms two morphotypes. *Experimental Mycology, 15* (1), 1-10. doi:https://doi.org/10.1016/0147-5975(91)90002-U

Barati, B., Gan, S. Y., Lim, P. E., Beardall, J., & Phang, S. M. (2019). Green algal molecular responses to temperature stress.*Acta Physiologiae Plantarum, 41* (2), 26. doi:10.1007/s11738-019-2813-1

Beck, A., Kasalicky, T., & Rambold, G. (2002). Myco-photobiontal selection in a Mediterranean cryptogam community with *Fulgensia fulgida* . *New Phytologist, 153* (2), 317-326. doi:https://doi.org/10.1046/j.0028-646X.2001.00315.x

Blot, M., Meyer, J., & Arber, W. (1991). Bleomycin-resistance gene derived from the transposon Tn5 confers selective advantage to*Escherichia coli* K-12. *Proceedings of the National Academy of Sciences, 88* (20), 9112-9116.

Bowman, S. M., & Free, S. J. (2006). The structure and synthesis of the fungal cell wall. *BioEssays, 28* (8), 799-808. doi:https://doi.org/10.1002/bies.20441

Bray, N. L., Pimentel, H., Melsted, P., & Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology, 34* (5), 525-527. doi:10.1038/nbt.3519

Calhoun, L. N., & Kwon, Y. M. (2011). Structure, function and regulation of the DNA-binding protein Dps and its role in acid and oxidative stress resistance in *Escherichia coli* : a review.*Journal of Applied Microbiology, 110* (2), 375-386. doi:10.1111/j.1365-2672.2010.04890.x

Calzadilla, P. I., Vilas, J. M., Escaray, F. J., Unrein, F., Carrasco, P., & Ruiz, O. A. (2019). The increase of photosynthetic carbon assimilation as a mechanism of adaptation to low temperature in*Lotus japonicus* . *Scientific Reports, 9* (1), 863. doi:10.1038/s41598-018-37165-7

Casano, L. M., del Campo, E. M., Garcia-Breijo, F. J., Reig-Arminana, J., Gasulla, F., Del Hoyo, A., Guera, A. & Barreno, E. (2011). Two Trebouxia algae with different physiological performances are ever-present in lichen thalli of *Ramalina farinacea* . Coexistence versus competition? *Environmental Microbiology, 13* (3), 806-818. doi:10.1111/j.1462-2920.2010.02386.x

Chavarria-Pizarro, T., Resl, P., Janjic, A., & Werth, S. (2022). Gene expression responses to thermal shifts in the endangered lichen*Lobaria pulmonaria* . *Molecular Ecology, 31* (3), 839-858. doi:https://doi.org/10.1111/mec.16281

Cornejo, C., Derr, C., & Dillman, K. (2017). *Ricasolia amplissima* (Lobariaceae): one species, three genotypes and a new taxon from south-eastern Alaska. *The Lichenologist, 49* (6), 579-596. doi:10.1017/S002428291700041X

Cruz-Loya, M., Kang, T. M., Lozano, N. A., Watanabe, R., Tekin, E., Damoiseaux, R., Savage V. M. & Yeh, P. J. (2019). Stressor interaction networks suggest antibiotic resistance co-opted from stress responses to temperature. *The ISME Journal, 13* (1), 12-23. doi:10.1038/s41396-018-0241-7

Culberson, W. L. (1986). Chemistry and sibling speciation in the lichen-forming fungi: Ecological and biological considerations.*The Bryologist, 89* (2), 123-131. doi:10.2307/3242752

del Hoyo, A., Alvarez, R., del Campo, E. M., Gasulla, F., Barreno, E., & Casano, L. M. (2011). Oxidative stress induces distinct physiological responses in the two *Trebouxia* phycobionts of the lichen*Ramalina*

*farinacea* . *Annals of Botany, 107* (1), 109-118. doi:10.1093/aob/mcq206

Demmig-Adams, B., Adams, W. W., Green, T. G. A., Czygan, F.-C., & Lange, O. L. (1990). Differences in the susceptibility to light stress in two lichens forming a phycosymbiodeme, one partner possessing and one lacking the xanthophyll cycle. *Oecologia, 84* (4), 451-456.

Domozych, D., Ciancia, M., Fangel, J., Mikkelsen, M., Ulvskov, P., & Willats, W. (2012). The cell walls of green algae: A journey through evolution and diversity. *Frontiers in Plant Science, 3* , 82.

Dubin, M. J., Mittelsten Scheid, O., & Becker, C. (2018). Transposons: A blessing curse. *Current Opinion in Plant Biology, 42* , 23-29. doi:10.1016/j.pbi.2018.01.003

Dumas, P., Bergdoll, M., Cagnon, C., & Masson, J.-M. (1994). Crystal structure and site-directed mutagenesis of a bleomycin resistance protein and their significance for drug sequestering. *The EMBO Journal, 13* (11), 2483-2492.

Elliott, T. (1998). Stress Proteins. In P. J. Delves (Ed.),*Encyclopedia of Immunology (Second Edition)* (pp. 2228-2232). Oxford: Elsevier.

Elvebakk, A., Papaefthimiou, D., Robertsen, E. H., & Liaimer, A. (2008). Phylogenetic patterns among *Nostoc* cyanobionts within bi- and tripartite lichens of the genus *Pannaria* . *Journal of Phycology, 44* (4), 1049-1059. doi:https://doi.org/10.1111/j.1529-8817.2008.00556.x

Ertz, D., Guzow-Krzemińska, B., Thor, G., Łubek, A., & Kukwa, M. (2018). Photobiont switching causes changes in the reproduction strategy and phenotypic dimorphism in the Arthoniomycetes. *Scientific Reports, 8* (1), 4952. doi:10.1038/s41598-018-23219-3

Farrar, J. F. (1976). Ecological physiology of the lichen*Hypogymnia physodes* . II. Effects of wetting and drying cycles and the concept of 'physiological buffering'. *New Phytologist, 77* (1), 105-113.

Fontaniella, B., Vicente, C., & Legaz, M.-E. (2000). The cryoprotective role of polyols in lichens: Effects on the redistribution of RNase in*Evernia prunastri* thallus during freezing. *Plant Physiology and Biochemistry, 38* (7), 621-627. doi:https://doi.org/10.1016/S0981-9428(00)00780-4

Fürtauer, L., Weiszmann, J., Weckwerth, W., & Nägele, T. (2019). Dynamics of plant metabolism during cold acclimation.*International Journal of Molecular Sciences, 20* (21), 5411. doi:10.3390/ijms20215411

Gagunashvili, A. N., & Andrésson, Ó. S. (2018). Distinctive characters of *Nostoc* genomes in cyanolichens. *BMC Genomics, 19* (1), 434. doi:10.1186/s12864-018-4743-5

Garcia-Rubio, R., de Oliveira, H. C., Rivera, J., & Trevijano-Contador, N. (2020). The fungal cell wall: *Candida* , *Cryptococcus* , and *Aspergillus* species. *Frontiers in Microbiology, 10* . doi:10.3389/fmicb.2019.02993

Gastebois, A., Aimanianda, V., Bachellier-Bassi, S., Nesseir, A., Firon, A., Beauvais, A., . . . Mouyna, I. (2013). SUN proteins belong to a novel family of β-(1,3)-glucan-modifying enzymes involved in fungal morphogenesis. *The Journal of Biological Chemistry, 288* (19), 13387-13396. doi:10.1074/jbc.M112.440172

Gauslaa, Y., Coxson, D. S., & Solhaug, K. A. (2012). The paradox of higher light tolerance during desiccation in rare old forest cyanolichens than in more widespread co-occurring chloro- and cephalolichens. *New Phytologist, 195* (4), 812-822. doi:https://doi.org/10.1111/j.1469-8137.2012.04221.x

Gazzano, C., Favero-Longo, S. E., Iacomussi, P., & Piervittori, R. (2013). Biocidal effect of lichen secondary metabolites against rock-dwelling microcolonial fungi, cyanobacteria and green algae.*International Biodeterioration & Biodegradation, 84* , 300-306. doi:https://doi.org/10.1016/j.ibiod.2012.05.033

Geoghegan, I., Steinberg, G., & Gurr, S. (2017). The role of the fungal cell wall in the infection of plants. *Trends in Microbiology, 25* (12), 957-967. doi:10.1016/j.tim.2017.05.015

Ghimire, S., Tang, X., Zhang, N., Liu, W., Qi, X., Fu, X., & Si, H. (2020). Genomic analysis of the SUMO-conjugating enzyme and genes under abiotic stress in potato (*Solanum tuberosum* L.).*International Journal of Genomics, 2020* , 9703638. doi:10.1155/2020/9703638

Goffinet, B., & Hastings, R. I. (1994). *The Lichen Genus Peltigera (Lichenized Ascomycetes) in Alberta* . Edmonton, Alberta: Provincial Museum of Alberta.

Goward, T., Goffinet, B., & Vitikainen, O. (1995). Synopsis of the genus *Peltigera* (lichenized Ascomycetes) in British Columbia, with a key to the North American species. *Canadian Journal of Botany, 73* (1), 91-111. doi:10.1139/b95-012

Green, T. G. A., Büdel, B., Heber, U., Meyer, A., Zellner, H., & Lange, O. L. (1993). Differences in photosynthetic performance between cyanobacterial and green algal components of lichen photosymbiodemes measured in the field. *New Phytologist, 125* (4), 723-731. doi:https://doi.org/10.1111/j.1469-8137.1993.tb03921.x

Green, T. G. A., Schlensog, M., Sancho, L. G., Winkler, J. B., Broom, F. D., & Schroeter, B. (2002). The photobiont determines the pattern of photosynthetic activity within a single lichen thallus containing cyanobacterial and green algal sectors (photosymbiodeme).*Oecologia, 130* (2), 191-198. doi:10.1007/s004420100800

Grzesiak, J., Woltyńska, A., Zdanowski, M. K., Górniak, D., Świątecki, A., Olech, M. A., & Aleksandrzak-Piekarczyk, T. (2021). Metabolic fingerprinting of the Antarctic cyanolichen *Leptogium puberulum* – associated bacterial community (Western Shore of Admiralty Bay, King George Island, Maritime Antarctica). *Microbial Ecology, 82* (3), 818-829. doi:10.1007/s00248-021-01701-2

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., . . . Regev, A. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols, 8* (8), 1494-1512. doi:10.1038/nprot.2013.084

Hájek, J., Váczi, P., Barták, M., Smejkal, L., & Lipavská, H. (2009). Cryoproective role of ribitol in *Xanthoparmelia somloensis* .*Biologia Plantarum, 53* (4), 677-684. doi:10.1007/s10535-009-0122-z

Hale, M. E. (1957). The *Lobaria amplissima -L. quercizans*complex in Europe and North America. *The Bryologist, 60* (1), 35-39. doi:10.2307/3240051

hbctraining, DGE_workshop (2022). *GitHub repository* , [accessed 05.04.2022]; https://github.com/hbctraining/DGE_workshop.

Henskens, F. L., Green, T. G. A., & Wilkins, A. (2012). Cyanolichens can have both cyanobacteria and green algae in a common layer as major contributors to photosynthesis. *Annals of Botany, 110* (3), 555-563. doi:10.1093/aob/mcs108

Hill, D. J. (1972). The movement of carbohydrate from the alga to the fungus in the lichen *Peltigera polydactyla* . *New Phytologist, 71* (1), 31-39.

Hill, D. J. (1985). Changes in Photobiont Dimensions and Numbers During Co-Development of Lichen Symbionts. In D. H. Brown (Ed.), *Lichen Physiology and Cell Biology* (pp. 303-317). Boston, MA: Springer US.

Hill, D. J., & Ahmadjian, V. (1972). Relationship between carbohydrate movement and the symbiosis in lichens with green algae. *Planta, 103* (3), 267-277.

Hitch, C. J. B., & Millbank, J. W. (1975). Nitrogen metabolism in lichens. VII. Nitrogenase activity and heterocyst frequency in lichens with blue-green phycobionts. *New Phytologist, 75* (2), 239-244.

Hoiczyk, E., & Hansel, A. (2000). Cyanobacterial cell walls: news from an unusual prokaryotic envelope. *Journal of Bacteriology, 182* (5), 1191-1199. doi:10.1128/JB.182.5.1191-1199.2000

Holtan-Hartwig, J. (1993). The lichen genus *Peltigera* , exclusive of the *P. canina* group, in Norway. *Sommerfeltia, 15* . doi:10.1017/S0024282994000150

Holtman, D. F. (1947). Antibiotic products of fungi. *The Botanical Review, 13* (2), 59-91. doi:10.1007/BF02861543

Holzinger, A., & Karsten, U. (2013). Desiccation stress and tolerance in green algae: consequences for ultrastructure, physiological and molecular mechanisms. *Frontiers in Plant Science, 4* , 327.

Honegger, R. (1984). Cytological aspects of the mycobiont–phycobiont relationship in lichens: Haustorial types, phycobiont cell wall types, and the ultrastructure of the cell surface layers in some cultured and symbiotic myco-and phycobionts. *The Lichenologist, 16* (2), 111-127. doi:10.1017/S0024282984000293

Honegger, R. (1985). Fine Structure of Different Types of Symbiotic Relationships in Lichens. In D. H. Brown (Ed.), *Lichen Physiology and Cell Biology* (pp. 287-302). Boston, MA: Springer US.

Honegger, R. (1986). Ultrastructural studies in lichens. II. Mycobiont and photobiont cell wall surface layers and adhering crystalline lichen products in four Parmeliaceae. *New Phytologist, 103* (4), 797-808.

Honegger, R. (2012). 15 The symbiotic phenotype of lichen-forming ascomycetes and their endo- and epibionts. In B. Hock (Ed.),*Fungal Associations* (pp. 287-339). Berlin, Heidelberg: Springer Berlin Heidelberg.

Honegger, R., & Brunner, U. (1981). Sporopollenin in the cell walls of*Coccomyxa* and *Myrmecia* phycobionts of various lichens: An ultrastructural and chemical investigation. *Canadian Journal of Botany, 59* , 2713-2734. doi:10.1139/b81-322

Hopke, A., Brown, A. J. P., Hall, R. A., & Wheeler, R. T. (2018). Dynamic fungal cell wall architecture in stress adaptation and immune evasion. *Trends in Microbiology, 26* (4), 284-295. doi:10.1016/j.tim.2018.01.007

Hu, X., Lu, Z., Shen, Y.-M., Tao, Y., & Song, S.-Y. (2019). Para-aminobenzoic acid synthase from mushroom *Agaricus bisporus*enhances UV-C tolerance in *Arabidopsis* by reducing oxidative DNA damage. *Acta Physiologiae Plantarum, 41* (9), 160. doi:10.1007/s11738-019-2936-4

Huang, B.-H., Lin, Y.-C., Huang, C.-W., Lu, H.-P., Luo, M.-X., & Liao, P.-C. (2018). Differential genetic responses to the stress revealed the mutation-order adaptive divergence between two sympatric ginger species.*BMC Genomics, 19* (1), 692. doi:10.1186/s12864-018-5081-3

Huson, D. H., Auch, A. F., Qi, J., & Schuster, S. C. (2007). MEGAN analysis of metagenomic data. *Genome Research, 17* (3), 377-386. doi:10.1101/gr.5969107

Hyvärinen, M., Härdling, R., & Tuomi, J. (2002). Cyanobacterial lichen symbiosis: The fungal partner as an optimal harvester. *Oikos, 98* (3), 498-504. doi:https://doi.org/10.1034/j.1600-0706.2002.980314.x

Ikeda, K., Nakayashiki, H., Takagi, M., Tosa, Y., & Mayama, S. (2001). Heat shock, copper sulfate and oxidative stress activate the retrotransposon MAGGY resident in the plant pathogenic fungus*Magnaporthe grisea* . *Molecular Genetics and Genomics, 266* (2), 318-325. doi:10.1007/s004380100560

Insarova, I. D., & Blagoveshchenskaya, E. Y. (2016). Lichen symbiosis: Search and recognition of partners. *Biology Bulletin, 43* (5), 408-418. doi:10.1134/S1062359016040038

Ivanov, A. G., Velitchkova, M. Y., Allakhverdiev, S. I., & Huner, N. P. A. (2017). Heat stress-induced effects of photosystem I: An overview of structural and functional responses. *Photosynthesis Research, 133* (1), 17-30. doi:10.1007/s11120-017-0383-x

Ivanov, D., Yaneva, G., Potoroko, I., & Ivanova, D. G. (2021). Contribution of cyanotoxins to the ecotoxicological role of lichens.*Toxins, 13* (5). doi:10.3390/toxins13050321

Jüriado, I., Kaasalainen, U., Jylhä, M., & Rikkinen, J. (2019). Relationships between mycobiont identity, photobiont specificity and ecological preferences in the lichen genus *Peltigera* (Ascomycota) in Estonia (northeastern Europe). *Fungal Ecology, 39* , 45-54. doi:10.1016/j.funeco.2018.11.005

Kaasalainen, U., Fewer, D. P., Jokela, J., Wahlsten, M., Sivonen, K., & Rikkinen, J. (2012). Cyanobacteria produce a high variety of hepatotoxic peptides in lichen symbiosis. *Proceedings of the National Academy of Sciences, 109* (15), 5886-5891. doi:10.1073/pnas.1200279109

Kaasalainen, U., Jokela, J., Fewer, D. P., Sivonen, K., & Rikkinen, J. (2009). Microcystin production in the tripartite cyanolichen*Peltigera leucophlebia* . *Molecular Plant-Microbe Interactions, 22* (6), 695-702. doi:10.1094/MPMI-22-6-0695

Kammerscheit, X., Chauvat, F., & Cassier-Chauvat, C. (2019). First in vivo evidence that glutathione-S-transferase operates in photo-oxidative stress in cyanobacteria. *Frontiers in Microbiology, 10* (1899). doi:10.3389/fmicb.2019.01899

Karas, V. O., Westerlaken, I., & Meyer, A. S. (2015). The DNA-binding protein from starved cells (Dps) utilizes dual functions to defend cells against multiple stresses. *Journal of Bacteriology, 197* (19), 3206-3215. doi:10.1128/JB.00475-15

Kato, N., Brooks, W., & Calvo Ana, M. (2003). The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by veA, a gene required for sexual development.*Eukaryotic Cell, 2* (6), 1178-1186. doi:10.1128/EC.2.6.1178-1186.2003

Keszenman, D. J., Candreva, E. C., & Nunes, E. (2000). Cellular and molecular effects of bleomycin are modulated by heat shock in*Saccharomyces cerevisiae* . *Mutation Research, 459* (1), 29-41. doi:https://doi.org/10.1016/S0921-8777(99)00056-7

Keszenman, D. J., Candreva, E. C., Sánchez, A. G., & Nunes, E. (2005). RAD6 gene is involved in heat shock induction of bleomycin resistance in*Saccharomyces cerevisiae* . *Environmental and Molecular Mutagenesis, 45* (1), 36-43. doi:10.1002/em.20083

Kono, M., Kon, Y., Ohmura, Y., Satta, Y., & Terai, Y. (2020). In vitro resynthesis of lichenization reveals the genetic background of symbiosis-specific fungal-algal interaction in *Usnea hakonensis* .*BMC Genomics, 21* (1), 671. doi:10.1186/s12864-020-07086-9

Kopke, K., Hoff, B., Bloemendal, S., Katschorowski, A., Kamerewerd, J., & Kück, U. (2013). Members of the *Penicillium chrysogenum* velvet complex play functionally opposing roles in the regulation of penicillin biosynthesis and conidiation. *Eukaryotic Cell, 12* (2), 299-310. doi:10.1128/EC.00272-12

Koriem, A. M., & Ahmadjian, V. (1986). An ultrastructural study of lichenized and cultured *Nostoc* photobionts of *Peltigera canina* , *Peltigera rufescens* , and *Peltigera spuria* .*Endocytobiosis and Cell Research, 3* , 65-78.

Kranner, I. (2002). Glutathione status correlates with different degrees of desiccation tolerance in three lichens. *New Phytologist, 154* (2), 451-460. doi:https://doi.org/10.1046/j.1469-8137.2002.00376.x

Kranner, I., Beckett, R., Hochman, A., & Nash III, T. H. (2008). Desiccation-tolerance in lichens: A review. *The Bryologist, 111* (4), 576-593. doi:10.1639/0007-2745-111.4.576

Kukwa, M., Kosecka, M., & Guzow-Krzemińska, B. (2020). One name – one fungus: The influence of photosynthetic partners on the taxonomy and systematics of lichenized fungi. *Acta Societatis Botanicorum Poloniae, 89* (3). doi:10.5586/asbp.89311

Kurepa, J., Walker, J. M., Smalle, J., Gosink, M. M., Davis, S. J., Durham, T. L., Sung, D.Y., & Vierstra, R. D. (2003). The small ubiquitin-like modifier (SUMO) protein modification system in*Arabidopsis* : Accumulation of SUMO1 and -2 conjugates is increased by stress. *Journal of Biological Chemistry, 278* (9), 6862-6872. doi:10.1074/jbc.M209694200

Lange, O. L., & Green, T. G. A. (2005). Lichens show that fungi can acclimate their respiration to seasonal changes in temperature.*Oecologia, 142* (1), 11-19. doi:10.1007/s00442-004-1697-x

Lange, O. L., Green, T. G. A., & Ziegler, H. (1988). Water status related photosynthesis and carbon isotope discrimination in species of the lichen genus *Pseudocyphellaria* with green or blue-green photobionts and in photosymbiodemes. *Oecologia, 75* (4), 494-501. doi:10.1007/BF00776410

Lange, O. L., Kilian, E., & Ziegler, H. (1986). Water vapor uptake and photosynthesis of lichens: Performance differences in species with green and blue-green algae as phycobionts. *Oecologia, 71* (1), 104-110. doi:10.1007/BF00377327

Leach, M. D., Stead, D. A., Argo, E., & Brown, A. J. P. (2011). Identification of sumoylation targets, combined with inactivation of SMT3, reveals the impact of sumoylation upon growth, morphology, and stress resistance in the pathogen *Candida albicans .Molecular Biology of the Cell, 22* (5), 687-702. doi:10.1091/mbc.E10-07-0632

Légeret, B., Schulz-Raffelt, M., Nguyen, H. M., Auroy, P., Beisson, F., Peltier, G., Blanc, G., & Li-Beisson, Y. (2016). Lipidomic and transcriptomic analyses of *Chlamydomonas reinhardtii* under heat stress unveil a direct route for the conversion of membrane lipids into storage lipids. *Plant, Cell & Environment, 39* (4), 834-847. doi:10.1111/pce.12656

Liang, W., Wang, L., Shi, J., Lei, X., Yang, J., Wu, S., & Chen, W. (2014). Differential expression of antioxidant proteins in the drought-tolerant cyanobacterium *Nostoc flagelliforme* under desiccation. *Plant OMICS, 7* , 205-212.

Lommen, P. W., Schwintzer, C. R., Yocum, C. S., & Gates, D. M. (1971). A model describing photosynthesis in terms of gas diffusion and enzyme kinetics. *Planta, 98* (3), 195-220. doi:10.1007/BF00387066

Love, M., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology, 15* (12), 550. doi:10.1186/s13059-014-0550-8

Lu, J., Magain, N., Miadlikowska, J., Coyle, J. R., Truong, C., & Lutzoni, F. (2018). Bioclimatic factors at an intrabiome scale are more limiting than cyanobiont availability for the lichen-forming genus*Peltigera* . *American Journal of Botany, 105* (7), 1198-1211. doi:10.1002/ajb2.1119

Lu, Z., Kong, X., Lu, Z., Xiao, M., Chen, M., Zhu, L., Shen, Y., Hu, X., & Song, S. (2014). Para-aminobenzoic acid (PABA) synthase enhances thermotolerance of mushroom *Agaricus bisporus* . *PLoS one, 9* (3), e91298. doi:10.1371/journal.pone.0091298

Magain, N., & Sérusiaux, E. (2014). Do photobiont switch and cephalodia emancipation act as evolutionary drivers in the lichen symbiosis? A case study in the Pannariaceae (Peltigerales). *PLoS one, 9* (2), e89876. doi:10.1371/journal.pone.0089876

Martínez, I., Burgaz, A. R., Vitikainen, O., & Escudero, A. (2003). Distribution patterns in the genus *Peltigera* Willd. *The Lichenologist, 35* (4), 301-323. doi:https://doi.org/10.1016/S0024-2829(03)00041-0

Morel, M., Ngadin, A. A., Droux, M., Jacquot, J.-P., & Gelhaye, E. (2009). The fungal glutathione S-transferase system. Evidence of new classes in the wood-degrading basidiomycete *Phanerochaete chrysosporium* . *Cellular and Molecular Life Sciences, 66* (23), 3711-3725. doi:10.1007/s00018-009-0104-5

Müller, W. H., van der Krift, T. P., Krouwer, A. J., Wösten, H. A., van der Voort, L. H., Smaal, E. B., & Verkleij, A. J. (1991). Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum* . *The EMBO Journal, 10* (2), 489-495.

Muñoz-López, M., & García-Pérez, J. L. (2010). DNA transposons: nature and applications in genomics. *Current Genomics, 11* (2), 115-128. doi:10.2174/138920210790886871

Nair, S., & Finkel, S. E. (2004). Dps protects cells against multiple stresses during stationary phase. *Journal of Bacteriology, 186* (13), 4192-4198. doi:10.1128/JB.186.13.4192-4198.2004

Negi, P., Rai, A. N., & Suprasanna, P. (2016). Moving through the stressed genome: Emerging regulatory roles for transposons in plant stress response. *Frontiers in Plant Science, 7* (1448). doi:10.3389/fpls.2016.01448

O'Brien, H. E., Miadlikowska, J., & Lutzoni, F. (2005). Assessing host specialization in symbiotic cyanobacteria associated with four closely related species of the lichen fungus *Peltigera* . *European Journal of Phycology, 40* (4), 363-378. doi:10.1080/09670260500342647

O'Brien, H. E., Miadlikowska, J., & Lutzoni, F. (2009). Assessing reproductive isolation in highly diverse communities of the lichen-forming fungal genus *Peltigera* . *Evolution, 63* (8), 2076-2086. doi:https://doi.org/10.1111/j.1558-5646.2009.00685.x

Ocampo-Friedmann, R., & Friedmann, E. I. (1993). Biologically active substances produced by antarctic cryptoendolithic fungi. *Antarctic Journal of the United States, 28* (5), 252-254.

Oei, A. L., Vriend, L. E. M., Crezee, J., Franken, N. A. P., & Krawczyk, P. M. (2015). Effects of hyperthermia on DNA repair pathways: one treatment to inhibit them all. *Radiation Oncology, 10* (165). doi:10.1186/s13014-015-0462-0

Onesti, S., Miller, A. D., & Brick, P. (1995). The crystal structure of the lysyl-tRNA synthetase (LysU) from *Escherichia coli .Structure, 3* (2), 163-176. doi:https://doi.org/10.1016/S0969-2126(01)00147-2

Palmqvist, K., Dahlman, L., Jonsson, A., & Nash, T. H. (2008). The Carbon Economy of Lichens. In T. H. Nash III (Ed.), *Lichen Biology* (2 ed., pp. 182-215). Cambridge: Cambridge University Press.

Pardo-De la Hoz, C. J., Magain, N., Lutzoni, F., Goward, T., Restrepo, S., & Miadlikowska, J. (2018). Contrasting symbiotic patterns in two closely related lineages of trimembered lichens of the genus*Peltigera* . *Frontiers in Microbiology, 9* (2770). doi:10.3389/fmicb.2018.02770

Patel, P. K., & Free, S. J. (2019). The genetics and biochemistry of cell wall structure and synthesis in *Neurospora crassa* , a model filamentous fungus. *Frontiers in Microbiology, 10* (2294). doi:10.3389/fmicb.2019.02294

Pawlowski, K., & Bergman, B. (2007). Plant Symbioses with*Frankia* and Cyanobacteria. In H. Bothe, S. J. Ferguson, & W. E. Newton (Eds.), *Biology of the Nitrogen Cycle* (pp. 165-178). Amsterdam: Elsevier.

Peredo, E. L., & Cardon, Z. G. (2020). Shared up-regulation and contrasting down-regulation of gene expression distinguish desiccation-tolerant from intolerant green algae. *Proceedings of the National Academy of Sciences, 117* (29), 17438. doi:10.1073/pnas.1906904117

Prudden, J., Perry, J. J. P., Arvai, A. S., Tainer, J. A., & Boddy, M. N. (2009). Molecular mimicry of SUMO promotes DNA repair. *Nature Structural & Molecular Biology, 16* (5), 509-516. doi:10.1038/nsmb.1582

Purvis, O. W. (2000). *Lichens* . London: Natural History Museum.

Ranković, B., & Mišić, M. (2008). The antimicrobial activity of the lichen substances of the lichens *Cladonia furcata* ,*Ochrolechia androgyna* , *Parmelia caperata* and*Parmelia conspresa* . *Biotechnology & Biotechnological Equipment, 22* (4), 1013-1016. doi:10.1080/13102818.2008.10817601

Richardson, D. H. S., & Smith, D. C. (1968). Lichen physiology. X. The isolated algal and fungal symbionts of *Xanthoria aureola .New Phytologist, 67* (1), 69-77.

Richardson, D. H. S., Smith, D. C., & Lewis, D. H. (1967). Carbohydrate movement between the symbionts of lichens. *Nature, 214* (5091), 879-882. doi:10.1038/214879a0

Sakaki, K., Tashiro, K., Kuhara, S., & Mihara, K. (2003). Response of genes associated with mitochondrial function to mild heat stress in yeast *Saccharomyces cerevisiae* . *The Journal of Biochemistry, 134* (3), 373-384. doi:10.1093/jb/mvg155

Sayers, E. W., Beck, J., Brister, J. R., Bolton, E. E., Canese, K., Comeau, D. C., . . . Ostell, J. (2020). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research, 48* (D1), D9-D16. doi:10.1093/nar/gkz899

Schrank, B. R., Aparicio, T., Li, Y., Chang, W., Chait, B. T., Gundersen, G. G., . . . Gautier, J. (2018). Nuclear ARP2/3 drives DNA break clustering for homology-directed repair. *Nature, 559* (7712), 61-66. doi:10.1038/s41586-018-0237-5

Schwendener, S. (1868). Ueber die Beziehungen zwischen Algen und Flechtengonidien. *Botanische Zeitung, 26* (18), 289-292.

Shrestha, G., & St. Clair, L. L. (2013). Lichens: a promising source of antibiotic and anticancer drugs. *Phytochemistry Reviews, 12* (1), 229-244. doi:10.1007/s11101-013-9283-7

Skult, H. (1997). Notes on the chemical and morphological variation of the lichen *Ophioparma ventosa* in East Fennoscandia. *Annales Botanici Fennici, 34* (4), 291-297.

Smith, D., Muscatine, L., & Lewis, D. (1969). Carbohydrate movement from autotrophs to heterotrophs in mutualistic symbiosis.*Biological Reviews, 44* (1), 17-85. doi:https://doi.org/10.1111/j.1469-185X.1969.tb00821.x

Smith, D. C. (1963). Studies in the physiology of lichens. IV. Carbohydrates in *Peltigera polydactyla* and the utilization of absorbed glucose. *New Phytologist, 62* (2), 205-216.

Song, Y., Zhao, J., Chen, J., Luo, Q., Yang, R., Xu, J., . . . Yan, X. (2018). Heat shock-induced metabolic conversion of membrane lipids, fatty acids and volatile organic compounds of *Pyropia haitanensis*under different heat shock time. *Phycological Research, 66* , 89-99. doi:doi: 10.1111/pre.12206

Spribille, T., Resl, P., Stanton, D. E., & Tagirdzhanova, G. (2022). Evolutionary biology of lichen symbioses. *New Phytologist, 234* , 1566–1582. doi:10.1111/nph.18048

Steinhäuser, S. S., Andrésson, Ó. S., Pálsson, A., & Werth, S. (2016). Fungal and cyanobacterial gene expression in a lichen symbiosis: Effect of temperature and location. *Fungal Biology, 120* (10), 1194-1208. doi:10.1016/j.funbio.2016.07.002

Sundberg, B., Ekblad, A., Näsholm, T., & Palmqvist, K. (1999). Lichen respiration in relation to active time, temperature, nitrogen and ergosterol concentrations. *Functional Ecology, 13* (1), 119-125. doi:https://doi.org/10.1046/j.1365-2435.1999.00295.x

Tarkowski, Ł. P., & Van den Ende, W. (2015). Cold tolerance triggered by soluble sugars: a multifaceted countermeasure. *Frontiers in Plant Science, 6* (203). doi:10.3389/fpls.2015.00203

The UniProt Consortium. (2021). UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Research, 49* (D1), D480-D489. doi:10.1093/nar/gkaa1100

Tønsberg, T., & Holtan-Hartwig, J. (1983). Phycotype pairs in*Nephroma* , *Peltigera* and *Lobaria* in Norway.*Nordic Journal of Botany, 3* (6), 681-688. doi:https://doi.org/10.1111/j.1756-1051.1983.tb01479.x

Vančurová, L., Muggia, L., Peksa, O., Řídká, T., & Škaloud, P. (2018). The complexity of symbiotic interactions influences the ecological amplitude of the host: A case study in *Stereocaulon* (lichenized Ascomycota). *Molecular Ecology, 27* (14), 3016-3033. doi:https://doi.org/10.1111/mec.14764

Wagner, S., Bader, M. Y., & Zotz, G. (2014). Physiological Ecology of Tropical Bryophytes. In D. T. Hanson & S. K. Rice (Eds.),*Photosynthesis in Bryophytes and Early Land Plants* (pp. 269-289). Dordrecht: Springer.

Werth, S. (2011). Biogeography and Phylogeography of Lichen Fungi and their Photobionts. In D. Fontaneto (Ed.), *Biogeography of Microscopic Organisms. Is Everything Small Everywhere?* (pp. 191-208). Cambridge: Cambridge University Press.

Werth, S., & Sork, V. L. (2014). Ecological specialization in *Trebouxia* (Trebouxiophyceae) photobionts of *Ramalina menziesii* (Ramalinaceae) across six range-covering ecoregions of western North America. *American Journal of Botany, 101* (7), 1127-1140. doi:https://doi.org/10.3732/ajb.1400025

Woitzik, D., Weckesser, J., & Jürgens, U. J. (1988). Isolation and characterization of cell wall components of the unicellular cyanobacterium *Synechococcus* sp. PCC 6307. *Journal of General Microbiology, 134* (3), 619-627. doi:https://doi.org/10.1099/00221287-134-3-619

Wu, X. R., Kenzior, A., Willmot, D., Scanlon, S., Chen, Z., Topin, A., . . . Folk, W. R. (2007). Altered expression of plant lysyl tRNA synthetase promotes tRNA misacylation and translational recoding of lysine. *The Plant Journal, 50* (4), 627-636. doi:https://doi.org/10.1111/j.1365-313X.2007.03076.x

Zhang, X., Wu, C., Hu, C., Li, Y., Sun, X., & Xu, N. (2020). Lipid remodeling associated with chitooligosaccharides-induced heat tolerance of marine macroalgae *Gracilariopsis lemaneiformis* . *Algal Research, 52* , 102113. doi:https://doi.org/10.1016/j.algal.2020.102113

Zhou, W., Ryan, J. J., & Zhou, H. (2004). Global analyses of sumoylated proteins in *Saccharomyces cerevisiae* . Induction of protein sumoylation by cellular stresses. *The Journal of Biological Chemistry, 279* (31), 32262-32268. doi:10.1074/jbc.M404173200

## Tables

**Table 1:** The five top differentially expressed genes of each organism and parameter in question with functional annotation. Shown are transcript ID, putative function, p-value from differential expression analysis with DESeq and indication of the parameter at which the gene was differentially expressed.

| **gene** | **function** | **p-value** |
|---|---|---|
| **Ascomycete genes / photomorph** | **Ascomycete genes / photomorph** | **Ascomycete genes** |
| TRINITY_DN48557_c0_g1 | Isopenicillin N synthetase | 2.27e-07 |
| TRINITY_DN47170_c11_g1 | SUN domain protein | 2.05e-06 |
| TRINITY_DN48101_c1_g1 | Glutathione S-transferase | 4.63e-06 |
| TRINITY_DN24613_c0_g1 | Galactonate dehydratase | 5.82e-06 |
| TRINITY_DN38054_c0_g1 | 14_3_3 domain-containing protein | 7.26e-06 |
| **Ascomycete genes / temperature** | **Ascomycete genes / temperature** | **Ascomycete genes** |
| TRINITY_DN44171_c0_g1 | Rad60-SLD domain-containing protein | 3.92e-16 |
| TRINITY_DN35635_c0_g1 | Mediator of RNA polymerase II transcription subunit | 8.82e-11 |
| TRINITY_DN35336_c0_g1 | Heat shock | 6.38e-11 |
| TRINITY_DN47703_c0_g12 | zf-CHCC domain-containing protein | 1.17e-10 |
| TRINITY_DN38239_c0_g1 | GATA-type domain-containing protein | 1.93e-10 |
| **Cyanobacterial genes / temperature** | **Cyanobacterial genes / temperature** | **Cyanobacterial gen** |
| TRINITY_DN46972_c1_g1 | Lysine–tRNA ligase | 4.85e-05 |
| TRINITY_DN47414_c2_g2 | DUF3155 domain-containing protein | 5.28e-05 |
| TRINITY_DN45318_c4_g4 | Uncharacterized protein | 0.000196 |
| TRINITY_DN46919_c0_g1 | Uncharacterized protein | 0.000218 |
| TRINITY_DN48190_c0_g1 | Chaperone protein DnaK | 0.000222 |
| **Green algal genes / temperature** | **Green algal genes / temperature** | **Green algal genes** |
| TRINITY_DN47627_c2_g1 | Chlorophyll a-b binding protein, chloroplastic | 5.65e-05 |
| TRINITY_DN46800_c0_g1 | SHSP domain-containing protein | 0.0002766 |
| TRINITY_DN43942_c0_g1 | Photosystem II oxygen evolving complex protein PsbQ | 0.0016676 |
| TRINITY_DN48593_c1_g2 | Ribulose bisphosphate carboxylase small subunit | 0.0027463 |
| TRINITY_DN109771_c0_g1 | PsbP domain-containing protein | 0.0032282 |

## Figure legends

**Figure 1.** Photosymbiodemes of the lichen Peltigera britannica. (A) green-algal tripartite lobes (apple green) emerge from a bipartite thallus (bluish-gray) that has a cyanobacterium as a photosynthetic partner; (B) small cyanobacterial lobes develop from the cephalodia of a tripartite thallus.

**Figure 2.** Experimental setup to study differential gene expression in Peltigera britannica photomorphs. Dark green parts represent the cyanomorph, light green parts the tripartite morph. Lichen thalli were exposed to 4 °C for five days (treatment "4 °C_1"), then to 4 °C for two weeks (treatment "4 °C_2"), then to 15 °C for two hours (treatment "15 °C"), and finally to 25 °C for two hours (treatment "25 °C"). After each step, pieces of thallus (ca. 1 cm2) of the cyano- and the tripartite morph were cut off from all four specimens for RNA extraction.

**Figure 3.** PCA plot for the overall gene expression variance of the mycobiont. The principal component of variance in the mycobiont was temperature-mediated rather than photomorph-mediated (circle = tripartite morph; triangle = cyanomorph), with clusters for low (4 °C_1; green and 4 °C_2; blue), medium (15 °C; orange) and high temperatures (25 °C; red).

**Figure 4.** (A) 200 most significantly differentially expressed fungal genes of the cyanomorph and the tripartite morph. Lighter shades show DEGs that could not be functionally annotated. 123 DEGs were assigned to the cyanomorph (teal color) and 77 to the tripartite morph (green color). (B) Temperature-mediated differential gene expression of mycobiont, chlorophytes and cyanobacteria (comparing 25 °C with 4 °C_1). Bars include the 200 most significantly differentially expressed genes of each organism. Red bars show genes upregulated at 25°C, blue bars genes downregulated at 25°C. Lighter shades show genes that could not be functionally annotated. 103 of the 200 most significantly differentially expressed fungal genes were downregulated at 25 °C, 97 were upregulated; in the photobionts, 199 genes were upregulated and one was downregulated at 25 °C, respectively.

**Figure 5.** Visualization of the main biological processes (BP) attributed to differentially expressed ascomycete genes of the cyanomorph and the tripartite morph of Peltigera britannica. Different BPs are shown with distinct colors; section sizes correspond to the number of genes associated with a GOterm. In the cyanomorph, the main BPs were oxidation-reduction process (62%), protein carbohydrate metabolic process (18%) and protein phosphorylation (18%); a minor BP not shown in the graph is GPI anchor biosynthetic process. In the tripartite morph, the majority of fungal DEGs was annotated to oxidation-reduction process (60%), followed by transmembrane transport (32%), tricarboxylic acid cycle (1.55%) and phospholipid biosynthesis (1.49%). Minor BPs of the tripartite morph not depicted are protein peptidyl-prolyl isomerization, threonyl-tRNA aminoacylation, inositol biosynthetic process and cellular potassium ion homeostasis. Analysis based on GO annotation (Bioconductor package topGO).

**Figure 6.** Expression of stress proteins in the three lichen symbiosis partners based on the counts of the corresponding genes at each temperature. In the mycobiont (orange), upregulation of stress proteins begins at 15 °C already and it shows high stress at 25 °C; the same is true for the cyanobacteria (teal), although a higher number of stress related genes are expressed at lower temperatures (4 °C_1 and 4 °C_2) than in the mycobiont. Chlorophytes (green) appear to be stressed mainly at 25 °C.

**Hosted file**

`Figure 1.tif` available at https://authorea.com/users/499910/articles/580872-symbiont-specific-responses-to-environmental-cues-in-a-threesome-lichen-symbiosis