Stressed out: The effects of heat stress and parasitism on gene expression of the lichen-forming fungus *Lobaria pulmonaria*

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Abstract: Gene expression variation can be partitioned onto different components – regulatory, genetic and acclimatory effects, but for lichen-forming fungi, the relative importance of each of these effects is unclear. Here, we studied gene expression in the lichen-forming fungus *Lobaria pulmonaria* in response to thermal stress and parasitism by the lichenicolous fungus *Plectocarpon lichenum*. Our experimental procedure was to acclimate lichens thalli during three weeks to 4 °C and then expose them to 15 °C and 25 °C for 2 hours each, sampling infected and visually asymptomatic thalli at each temperature. Quantitative Real-Time PCR was utilized to quantify gene expression of six candidate genes, normalizing expression values with two reference genes. We found that two genes encoding heat shock proteins (*hsp88* and *hsp98*), two polyketide synthase genes (*rPKS1*, *nrPKS3*) and elongation factor 1-1-α (*efa*) were upregulated at higher temperatures. Moreover, we observed higher expression of *hsp98* at 25 °C in samples infected by *P. lichenum* than in uninfected samples. Finally, in partial redundancy analyses most of the explained variation in gene expression was related to temperature treatment; genetic variation and long-term acclimatization to sites contributed far less. Hence, regulatory effects, i.e. direct adjustments of gene expression in response to the temperature change, dominated over genetic and acclimatory effects in the gene expression variability of *L. pulmonaria*. This study suggests that *L. pulmonaria* could become a valuable lichen model for studying heat shock protein responses *in vivo*.

Keywords: transcriptome; thermal stress; lichenicolous fungi; polyketide synthase genes (PKS); heat-shock genes; stress response; quantitative Real-Time PCR (qPCR); acclimatation; acclimation

Introduction

Throughout the history of life, organisms have been challenged to survive in habitats that are

not stable, but subjected to fluctuations in important abiotic conditions such as temperature, humidity, pH and UV-light (MacKenzie *et al.* 2001; MacKenzie *et al.* 2004; Evans *et al.* 2013; Hamann *et al.* 2016). In order to deal with those changing conditions, the ability to regulate the expression of stress-related genes is vital (Evans et al. 2013). Investigations of both eukaryotes and prokaryotes have shown that gene expression plays a crucial role for the tolerance of extreme conditions such as drought (Wang *et al.* 2015; Carmo *et al.* 2019), temperature and salinity stress (Jamil *et al.* 2011; Che *et al.* 2013; Zhang *et al.* 2017), as well as exposure to toxins (Whitehead *et al.* 2010). Understanding the mechanisms and different pathways of this gene-expression response to stressful conditions, is important for getting better insights in survival mechanisms and the interplay of organisms with their environment. Environmental stress response has been subject to various studies of many different organisms (Mizoguchi *et al.* 1997; Gasch 2007; Dixon *et al.* 2020; Terhorst *et al.* 2020). For fungi, environmental stress response was first described in *Saccharomyces cerevisiae* (Gasch *et al.* 2000). Stress genes play an important role in carbohydrate metabolism, response to oxidative stress, intracellular signaling, DNA-damage repair and protein metabolism, especially protein folding (Gasch 2007.; O'Meara *et al.* 2019).

One common environmental stressor organisms are confronted with is thermal stress (Arshad *et al.* 2017). In most habitats, organisms have to deal with more or less rapidly changing temperatures, but responses to thermal stress have become an important issue also due to the rapid increase of temperatures and higher fluctuations and extremes because of global climate change (IPCC 2021). Global mean surface temperatures will continue increase in the first half of the 21st century, with the amount of the increase depending on the quantity of future man-made CO_2-emissions (IPCC 2021). Heat shock response represents one of the important mechanisms for organisms to adapt to stressful conditions at the cellular level (O'Meara et al. 2019).

In response to environmental stress, gene expression needs to be regulated to a new cellular equilibrium to ensure cell survival. We hereafter refer to the variation in gene expression that is involved in keeping up cellular equilibrium under temperature stress as 'regulatory effects'. In order to respond to thermal stress and survive, organisms need to be able to sense heat and, as a response, conduct an adequate regulation of genes that might prevent or reduce the damage caused by high temperatures. In general, heat stress can be sensed through two effects: First, the accumulation of denatured proteins which results in the activation of a heat shock factor (Franzmann *et al.* 2008), and second, changes in thermosensitive structures like DNA, RNA, proteins or lipids that serve as primary sensors which can either have a direct effect or activate signal transduction pathways such as the very conserved mitogen-activated kinase (MAPK) pathways which are important in the stress responses of filamentous ascomycetes (Hagiwara *et al.* 2016). While the first reaction initiated by these signaling pathways can include fast responses such as the use of previously synthesized proteins or the regulation of channels and transporters, the main heat shock response is carried out through gene regulation leading to a major change in transcriptional patterns after a few minutes (Albrecht *et al.* 2010; Roncarati and Scarlato 2017). Many genes, for example those involved in cell-cycle, RNA metabolism and synthesis of proteins, are simultaneously downregulated under stress conditions, some reaching several maxima in expression over a time course of two hours or fluctuating over time (Albrecht et al. 2010; de Nadal *et al.* 2011; Takahashi *et al.* 2017).

An important reaction to thermal stress is the expression of genes encoding heat shock proteins (HSPs). HSPs are able to un- and refold proteins which became misfolded because of heat exposure (Albrecht et al. 2010). The heat-induced upregulation of HSPs has been shown in many organisms including prokaryotes and eukaryotes, revealing many HSP families that interact and regulate each other in different pathways (Plesofsky-Vig and Brambl 1998; Miot

92 *et al.* 2011; Smith *et al.* 2012; Li and Buchner 2013; Park *et al.* 2015). The heat shock protein
93 gene *hsp88* of an entomopathogenic fungus has been cloned and characterized by Park et al.
94 (2014). Under thermal stress, *hsp88* was 15-55-fold upregulated in the lichen-forming fungus
95 *Peltigera membranacea* (Steinhäuser *et al.* 2016). An important heat shock protein gene in *A.*
96 *fumigatus* is *hsp98* (Do *et al.* 2009), and this gene was upregulated under thermal stress in
97 *Peltigera membranacea* (Steinhäuser et al. 2016).
98 While the increased expression of heat shock protein genes is a universal and well-
99 known reaction to environmental stressors, another reaction that could possibly be linked to
100 stressful conditions is the production of polyketides in fungi (Timsina *et al.* 2013).
101 Polyketides are secondary metabolites featuring antimicrobial, antitumor,
102 immunosuppressive, antifungal and antiparasitic properties and they are therefore not only of
103 great relevance for pharmaceutical purposes (Nivina *et al.* 2019), but also of interest for
104 physiological and ecological questions. Polyketides have been suspected to protect organisms
105 from environmental stresses such as high light levels, drought, or from herbivory and fungal
106 parasites (Lawrey 1986; Lawrey 1989; Torzilli *et al.* 1999; Gauslaa and McEvoy 2005;
107 Timsina et al. 2013). The biosynthesis of polyketides out of 2-, 3- or 4-carbon compounds is
108 catalyzed by polyketide synthases (PKSs), which are large multi-enzyme systems with a
109 molecular weight up to 10 000 kDa (Khosla *et al.* 1999). Type I PKSs are large proteins
110 consisting of several functional domains and type III PKSs are simpler enzymes catalyzing
111 the formation of a product within a single active site (Nivina et al. 2019). Non-reducing PKSs
112 characteristically catalyze the synthesis of aromatic polyphenols but fungal reducing PKSs
113 reduce beta-carbons with different domains to form reduced aromatic rings or aliphatic rings,
114 e.g. macrolides (Bertrand and Sorensen 2018). Generally, there is a connection between
115 polyketide production in lichens and abiotic conditions such as nutrient supply, substrate pH
116 and light, with the production being higher under stressful conditions and negatively

117 correlated with growth (Armaleo *et al.* 2008; Timsina et al. 2013). Thus, it is conceivable that
118 heat stress would lead to an upregulation of polyketide synthase genes, causing a
119 corresponding increase of polyketide production. In the lichen-forming fungus *Lobaria*
120 *pulmonaria* (L.) Hoffm. (lichenized ascomycetes, Peltigerales), three major carbon-based
121 secondary compounds are produced by PKS genes: stictic, constictic, and norstictic acid, as
122 well as some chemically related minor compounds (Bidussi *et al.* 2013; Gauslaa *et al.* 2013).
123 The depsidones norstictic and stictic acid are produced via the acetate-polymalonate pathway
124 (Ranković and Kosanić 2019). Some lichen secondary compounds including those of *L.*
125 *pulmonaria* have anti-herbivore and antibiotic properties (Suleyman *et al.* 2003; Asplund and
126 Gauslaa 2008; Nybakken *et al.* 2010). Some secondary compounds such as lecanoric acid
127 may also have antifungal properties, preventing lichen colonization by certain lichenicolous
128 fungi (Lawrey 1989; Lawrey 2000; Lawrey and Diederich 2003), and some may be useful as
129 anti-cancer drugs (Shrestha and St. Clair 2013; Dar *et al.* 2021; Yang *et al.* 2021).
130 The lichenicolous fungus *Plectocarpon lichenum* (Sommerf.) D. Hawksw. forms
131 conspicuous darkish brown structures on thalli of *Lobaria pulmonaria*; these structures
132 represent stromata made of hyphae of the lichenicolous fungus and of its lichen host
133 (Bergmann and Werth 2017). A recent study based on qPCR found that the mycelium of this
134 lichenicolous fungus is localized mainly in the stromata, with only a very low signal being
135 detected directly adjacent to galls (Bergmann and Werth 2017). Areas including stromata
136 have on average a two-fold biomass when compared to adjacent asymptomatic thallus parts,
137 and thalli infected by *P. lichenum* most often contain many stromata (Bergmann and Werth
138 2017). Thus, it is conceivable that *P. lichenum* taps substantially into the overall carbon pool
139 of *L. pulmonaria*. Thalli of *Lobaria pulmonaria* infected by *Plectocarpon lichenum* were
140 found to have a significantly reduced amount of carbon-based secondary compounds
141 (Asplund *et al.* 2016). Similarly, in *Lobarina scrobiculata* (Scop.) Nyl. ex Cromb., polyketide

concentration was reduced to less than half in thalli infected by the lichenicolous fungus *Plectocarpon scrobiculatae* Diederich & Etayo, when compared to uninfected thalli (Merinero *et al.* 2015). Either, infections by *Plectocarpon* lead to an overall downregulation of PKS genes in the parasitized thalli, or the lichenicolous fungi might degrade the lichen's secondary compounds with extracellular enzymes (Lawrey 2000). The first hypothesis can be tested by an analysis of differential expression of PKS genes.

Abiotic conditions such as different habitats can also influence gene expression (e.g. MacFarlane and Kershaw 1980; Cheviron *et al.* 2008; Whitehead et al. 2010; Steinhäuser et al. 2016). Habitat-related differential gene expression could be composed of both genetic and acclimatory factors (Cheviron et al. 2008; Whitehead et al. 2010; Palumbi *et al.* 2014). If the differences in gene expression are caused by long-term physiological acclimatization effects, they should vanish after acclimation to common conditions in the laboratory, or in a common garden experiment. Lichen populations grown in the lab or a common garden can however retain site-specific differences in gene expression (Steinhäuser et al. 2016) or physiological state (MacFarlane and Kershaw 1980; Schipperges *et al.* 1995). These studies suggest that there might be a substantial genetic component to variation in gene expression. However, the relative importance of the genetic component has not yet been scrutinized.

The main aim of this study was to obtain a better understanding of gene expression variation in response to increased temperatures and its partitioning into different factors in the lichen-forming fungus *L. pulmonaria*. At the onset of our study, it was not known at which temperature heat shock is induced in *L. pulmonaria*. So, first, we investigated the expression patterns of *L. pulmonaria* heat shock protein and polyketide synthase genes exposed to different temperatures to quantify the regulatory component of gene expression variation. The specific question we asked was, does thermal stress caused by a temperature increase from 4 °C to 15 °C and then to 25 °C result in differential expression of heat shock protein and

polyketide synthase genes?

Given that earlier studies indicated that the concentration of lichen secondary metabolites was reduced in *Lobaria pulmonaria* thalli parasitized by *P. lichenum* (Asplund et al. 2016; 2018), we hypothesized that presence of the lichenicolous fungus *P. lichenum* would have an effect on the expression of polyketide synthase genes, leading to their down-regulation (biotic component of gene expression variation). However, since polyketide production may increase due to environmental stress, we expected higher gene expression in polyketide synthases under thermal stress conditions.

Furthermore, we examined whether physiological long-term acclimatization had a long-lasting effect on the physiological state of individuals, persisting as collecting-site related differences even after acclimation to common laboratory conditions (acclimatory component). To address this issue in our study, we compared thalli of *L. pulmonaria* from a population in Austria with one in Tenerife after acclimating them to common laboratory conditions. Finally, we related gene expression variation to genetic distance to quantify the genetic component of gene expression variation. To assess the relative roles of the regulatory, acclimatory, biotic, and genetic components of gene expression variation, a variance partitioning approach was used.

Materials and methods

Collection of lichen samples

Samples were collected in February 2015 from a site in Austria (AU7) and a site in Tenerife (ST7). AU7 was chosen as one of four populations of *Lobaria pulmonaria* described in the literature, located in the Ennstaler Alps at Tamischbachgraben (47°38'N 14°41'E) at about 700 m above sea level (Scheidegger *et al.* 2012). Five thalli (AU7-01 – AU7-05) of similar size were collected from trunks of Sycamore maple (*Acer pseudoplatanus* L.). In order to collect different genotypes, they were taken from trees at a distance of at least 20 m. Site ST7

was located in a pine (*Pinus canariensis* C.Sm. ex DC.) forest in Tenerife, Canary Islands (N28°24.51096', W16°25.06404', 1560 m a.s.l.); this site frequently received fog. From this site, ten thalli with *Plectocarpon lichenum* (ST7-11 – ST7-20) and ten without (ST7-01 – ST7-10) were gathered. Samples with *Plectocarpon* infection contained stromata visible to the naked eye. Samples were collected at a distance of at least 10 m from each other. All thalli were stored dry and in darkness at a temperature of about 4 °C for five days until the beginning of the experiment.

Acclimation phase and temperature treatment

The thalli were placed in petri dishes lined with filter paper, which was previously rinsed with distilled water to create a neutral substratum for the lichens. In order to allow them to acclimate, the lichens were grown in a styrofoam box for three weeks in a cold room at 4 °C under constant light conditions of 62.4 lx (in the middle of the box) to 38.4 lx (on the edge of the box). To achieve as equal conditions as possible, the samples in the middle and on the edge were swapped periodically. They were watered frequently with dH_2O, however, allowing them to dry out every few days in order to avoid mold and to simulate the natural change of metabolically active and inactive phases due to re- and dehydration. At the end of the acclimation period at 4 °C, tissue samples were taken for RNA extractions from fully hydrated lobes by cutting off 5 × 5 mm pieces from the edge of each thallus and placing them in ice-cooled RNA stabilization solution (3.53 M ammonium sulfate, 16.7 mM sodium citrate, 13.3 mM EDTA, pH = 5.2) (De Wit *et al.* 2012). From the samples ST7-11 – ST7-20, only areas with no visible growth of *P. lichenum* were used for sampling as we were asking if presence of the lichenicolous fungus influenced gene expression of the entire thallus, rather than just the symptomatic thallus parts. Then, the light source and the lichen thalli were moved to a plant growth chamber in which the thalli were exposed to higher temperatures, first to 15 °C and then 25 °C. Each temperature treatment was kept for three hours prior to

tissue sampling as described above; this experimental setup was similar to the one used by Steinhäuser et al. (2016).

RNA extraction and reverse transcription to cDNA

The samples taken after exposure to 4 °C and 15 °C were immediately used for RNA extraction, while the 25 °C samples were stored overnight at -80 °C. Others have reported successful RNA extractions for *Lobaria pulmonaria* using Tri Reagent (Doering *et al.* 2014), and this is also the extraction chemistry that we used. Samples were homogenized in TRI Reagent (Sigma Aldrich) using Tissue Lyser II (Qiagen) with a 3 mm stainless steel polishing bead (Kugel Pompel, Austria). RNA extraction was performed using 2 ml Heavy-Gel Phase-Lock gel tubes (5 Prime) based on the protocol in Appendix C. RNA concentration was quantified using a P-Class NanoPhotometer (Implen). The RNA concentrations ranged from 120 to 620 ng/µL. To remove remaining genomic DNA from the samples, a digest of genomic DNA was performed with the RNase-Free DNase Set (Qiagen). The RNA was pipetted to a mix of DNase I, RDD buffer and RNase free water and then the mix was incubated in a thermocycler (AlphaMetrix Biotech) at 37 °C for 15 min and at 75 °C for 5 min. After the DNA digest, the RNA concentration was quantified again and all samples were diluted to the same concentration (100 ng/µL) in order to enable quantitative comparisons.

For cDNA synthesis, 20 µL of digested RNA were pipetted to a mix of 4 µL 10× RT random primers, 1.6 µL dNTP mix (4 mM each), 4 µL 10× RT buffer, 2 µL MultiScribe Reverse Transcriptase (100 U) and 8.4 µL RNase-free water, using reagents and protocols provided with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The assays were incubated at 25 °C for 10 min, at 37 °C for 120 min, at 85 °C for 5 min and then cooled to 4 °C. After the cDNA synthesis, the samples were diluted with 160 µL of RNase free H_2O in order to reach a final cDNA concentration of 10 ng/µL.

241 **Selection of genes**

242 As reference genes, we utilized two genes playing a vital role in metabolism which should
243 have constant expression between different temperatures, betatubulin (*bet*) and
244 glyceraldehyde 3-phosphate dehydrogenase (*gpd*); these genes had previously been validated
245 in other studies of lichen-forming fungi (Joneson *et al.* 2011; Miao *et al.* 2012).

246 For the candidate genes, on the one hand we focused on genes relevant under stressful
247 conditions, especially genes encoding heat shock proteins that are likely to change in
248 expression due to increasing temperatures. On the other hand, we chose reducing and non-
249 reducing types of polyketide synthase I. Table 1 shows the list of genes we considered as
250 reference or candidate genes with putative functions. A BLASTX analysis was performed to
251 verify the identity of loci (Altschul *et al.* 1997). Loci were selected based on PCR
252 amplification (specific amplification, i.e. a single amplicon) and qPCR results. The sequences
253 of all tested loci were deposited in GenBank (accessions KX866397-KX866407).

254 --- Table 1 ---

255 As candidate and reference genes, we considered only conserved regions of the genes based
256 on 454 genomic data of the mycobiont *Lobaria pulmonaria* (C. Scheidegger, unpublished
257 data). Further information on the 454 data is given in Werth *et al.* (2013); the multispore
258 mycobiont culture F2, which was used to obtain the data, is described in Widmer *et al.* (2010)
259 and Cornejo *et al.* (2015). Using partially sequenced mycobiont genomic data, we obtained
260 genomic sequences of heat shock protein and PKS genes of *L. pulmonaria* based on sequence
261 similarity with protein sequences and a DNA sequence from GenBank. The following protein
262 sequences were used to find *L. pulmonaria* sequences of PKS genes: ABV71377 (*L.*
263 *pulmonaria*), BAN29051 (*Lobaria orientalis* (Asahina) Yoshim.), ABV71378 (*L.*
264 *scrobiculata*), AEE87273, ADF28669, AEE87274, ADF28670, AEE65376, AEE65375,
265 AEE65373, ADF28668, AEE65377, AEE65374, AEE65372 (*Peltigera membranacea* (Ach.)

Nyl.), and a DNA sequence (EF363900, *L. pulmonaria*). The following GenBank accessions were used to find stress genes: ACV03836 (*Msn2*, *Aspergillus parasiticus* Speare), EDN02919 (*hsp88*, *Ajellomyces capsulatus* (Kwon-Chung) McGinnis & Katz), EDP56763 (heat shock protein gene *hsp98/hsp104/ClpA*, *Aspergillus fumigatus* Fresen.), EYE93161 (putative signal peptide peptidase, a gene involved in signal transduction in *Aspergillus ruber* Thom & Church), AAR30137 (putative histidine kinase *HHK2p*, *Fusarium verticillioides* (Sacc.) Nirenberg), and elongation factor 1-α (AFQ55277), which has been shown function as a molecular chaperone upregulated under heat conditions and salt stress in plants (Shin *et al.* 2009). Reference genes were obtained through sequences of β-tubulin (AFJ45056, *P. membranacea*) and glyceraldehyde 3-phosphate dehydrogenase (AFJ45057, *P. membranacea*). Only blast hits with an e-value < 10^{-40} were retained. After inspecting alignments, we selected genes with a high similarity to *hsp88*, *hsp98*, putative signal peptide peptidase, putative histidine kinase *HHK2p*, reducing and non-reducing types of *PKS I*, actin, β-tubulin, glyceraldehyde 3-phosphate dehydrogenase, and elongation factor 1-α.

The *Lobaria pulmonaria* Scotland v1.0 reference genome was released on JGI after we performed our experiment. To assess the correspondence of our gene set to NCBI gene models and annotations, we blasted each gene against the *Lobaria pulmonaria* genome on JGI MycoCosm (Table 1), link: https://genome.jgi.doe.gov/pages/blast-query.jsf?db=Lobpul1, date accessed: June 12, 2018.

Primer design and efficiency

To design primers, we focused on an amplicon length of about 100 to 200 base pairs and a primer length of 18 – 26 base pairs. The primers were designed using the NCBI Primer-BLAST software (Ye *et al.* 2012) and checked for melting point (optimum: 60 – 61°C) and self-complementarity (<5) with OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, USA). Primers were ordered from Microsynth (Balgach, Switzerland), diluted to a

concentration of 5 µM and were first tested in a normal PCR. PCR products were run on a 1 % agarose gel stained with Midori Green at 80 V for 20 min in 1× TAE buffer. Since not all of the primers amplified in a sufficient way, we chose those producing the best results and showing specific amplification (a single amplicon) for the final qPCR experiments. In accordance with the MIQE guidelines (Bustin *et al.* 2009), all final primers were tested for their efficiency using LinReg 11.0. LinReg performs a linear regression analysis with the raw data for all replicate reactions of a primer (including the amplification data from all 40 cycles of a qPCR run) and calculates the primer efficiency.

--- Table 2 ---

RT-qPCR procedure

The qPCR was performed using 96-well optical PCR plates and seals (LabConsulting) and KAPA SYBR FAST qPCR Kit Universal (KAPA Biosystems). Each well contained a total reaction volume of 10 µl, consisting of 5 µl 2× KAPA SYBR FAST qPCR Master Mix Universal, 0.2 µl 50× ROX Low, 2.8 µl nuclease free water, 250 nM of each forward and reverse primer, and 10 ng cDNA (1 µl). The qPCR was run on a 7500 Real-Time PCR System (Applied Biosystems). Cycling conditions were started with 3 min at 95 °C in order to activate the hot start polymerase, followed by 40 amplification cycles consisting of 15 s denaturation at 95 °C and 1 min annealing/extension at 60 °C.

The entire experiment was run once and at the end, material was harvested for RNA extractions. For each sample included in the qPCRs, we made a technical duplicate, which was preferentially run on the same plate. These technical duplicates used the same cDNA and were performed to account for pipetting error in the qPCR. We also ran at least two non-template controls (NTC) per locus on each plate in order to detect potential contaminations (NTCs with a cycle threshold (Ct) -value <34). Technical duplicates varying by more than 1 cycle in their Ct-values were repeated, except for those with a Ct-value >30, for which a

316 difference of more than 1 cycle is not unusual due to the low RNA-concentration.

317 **Processing of qPCR data**

318 Ct-values resulting from qPCR were standardized by the reference genes, and the resulting
319 values (ΔCt) were used for data analysis. The cycle threshold Ct stands for the number of
320 PCR-cycles necessary for the fluorescent signal of a sample to exceed a predefined threshold
321 (0.2), which allows a relative comparison of the original amount of cDNA copies of a gene.
322 The earlier on in a qPCR reaction the threshold cycle is reached, the higher was the initial
323 mRNA quantity. In order to minimize variation, we created the geometric mean of the Ct-
324 values of each technical duplicate and used it for further calculations (for simplification from
325 now on referred to as the Ct-value). Then, for each candidate gene in each individual, a ΔCt-
326 value was calculated according to the MIQE guidelines (Bustin et al. 2009). We subtracted
327 the geometric mean of the reference genes from the Ct-value of the candidate gene: $\Delta Ct = Ct_{candidate\ gene} - \text{geomean}\ (Ct_{reference\ gene\ 1}, Ct_{reference\ gene\ 2})$.
329 In order to illustrate differential gene expression, we then used the ΔCt to create the relative
330 expression (relative quantity = RQ) of each candidate gene. Here, we used the individual with
331 the lowest expression as reference sample and calculated a ΔΔCt, from which the relative
332 expression was calculated as follows: $\Delta\Delta Ct = \Delta Ct - \Delta Ct_{reference\ sample}$. $RQ = 2^{-\Delta\Delta Ct}$ (Pfaffl
333 2001). Using RQ, we created charts to allow a visual inspection of gene expression as a
334 function of temperature, site, and presence of *Plectocarpon*.

335 **Generation and processing of microsatellite data**

336 To investigate the genetic component of gene expression, microsatellite data were generated
337 so that genetic relationships among individuals could be inferred. For each individual used in
338 the experiment, microsatellite data for the loci *MS4*, *LPu37451*, *LPu28*, *LPu25*, *LPu09*,
339 *LPu23*, *LPu17457*, *LPu39912*, *LPu13707*, *LPu15* and *LPu04843* (Walser *et al.* 2003, Widmer
340 *et al.* 2010, Werth *et al.* 2013) were generated by Cecilia Ronnås at University of Graz and

genotyped by SW using the microsatellite plugin implemented in Geneious v. 6.1.6. Individual genetic distance calculation followed Kosman & Leonard (2005) and the BIONJ algorithm, an improved version of the neighbor-joining algorithm, was utilized to generate an unrooted tree (Gascuel 1997); these algorithms were implemented in the R packages 'PopgenReport' (Adamack and Gruber 2014) and 'ape' v. 3.3 (Paradis *et al.* 2004; Paradis 2006) and analyses were run in R version 4.0.2 (R Core Team 2018).

Data analysis

For each putative reference gene, stability of expression was assessed over all studied samples and experimental conditions using boxplots. Additionally, NormFinder v. 0953 (Andersen *et al.* 2004) was used to quantify the stability of expression for the reference genes. The NormFinder program identifies genes with optimal normalization among a set of candidate genes. The lowest stability value indicates the most stable expression within the gene set examined, having the least variation within and among groups. (Andersen et al. 2004)

Statistical analysis was performed in the R statistical environment, version 3.2.2 (R Core Team 2018). We tested for statistically significant differences in temperature and site using a multifactorial ANOVA of linear mixed effect models with temperature and site as fixed factors and individual as random factor. If statistical significance was found, Tukey's post-hoc tests were used to calculate the *p*-values for comparisons between the three temperatures and/or between sites. In order to eliminate unintended factors, from the ST7 population only individuals without *P. lichenum* were used for comparisons between sites. To examine the difference between individuals with and without *P. lichenum* within the ST7 population, Student's *t*-tests were applied.We partitioned the variance in gene expression by RDA onto temperature, site, genetic factors and *Plectocarpon* infection. First, a principal component analysis was performed on the microsatellite data to reduce their dimensionality. To do so, microsatellite alleles were coded as binary variables for each studied sample and

principal component analysis (PCA) was performed with the 'princomp' function in R version 4.0.2. A total of 10 PCA axes were retained, explaining 80% of the variation in the microsatellite data, and these were included in (partial) redundancy analyses which was implemented in the package 'vegan' 2.5.6 (Oksanen *et al.* 2016). The aim of the redundancy analysis was to determine how much of the variance in gene expression of *Lobaria pulmonaria* was explained by genetic vs. other factors (temperature, site, *Plectocarpon lichenum* infection).

Results

Verification of gene identities and of expression stability

As expected, the 454 DNA sequences of *Lobaria pulmonaria* used to design primers matched with parts of the *Lobaria pulmonaria* Scotland v. 1.0 reference genome with identities of 99-100% (Table 1). Our gene names matched the KOG descriptions in the annotations of the *L. pulmonaria* genome for *bet, efa,* and *gpd*. Moreover, as expected, *hsp88* and *hsp98* were chaperones according to the *Lobaria pulmonaria* Scotland genome annotation. The PKS genes were annotated as "fatty acid synthase and related" proteins in the *Lobaria pulmonaria* Scotland reference genome.

The efficiency of all primer pairs was ≥ 88% (Table 2). The stability values of *bet* and *gpd* was assessed with Normfinder software; they were were 0.014 and 0.015, hence these genes were were stable in expression.

Effects of *Plectocarpon lichenum* infection

Comparing the gene expression patterns of individuals with and without *P. lichenum* from site ST7, only in one gene a significant difference was found. While showing no difference in the 4 °C (Student's *t*-test: $p = 0.4084$, see Table 3) and 15 °C temperature treatments

391 (Student's *t*-test: $p = 0.8715$, see Table 3), expression of the heat shock protein gene *hsp98*
392 was significantly higher at 25 °C for individuals infected with *P. lichenum* (Student's *t*-test: p
393 = 0.0102). None of the other genes were differentially expressed between individuals with or
394 without *P. lichenum* (Student's *t*-test: $p > 0.1$; Fig. 1C).
395 --- Figure 1 ---
396 --- Table 3 ---
397 **Effects of temperature and collecting site**
398 In all genes tested, a significant difference in gene expression due to increased temperatures
399 was observed (ANOVA: $p < 0.009$, see Table 4, Fig. 1). There was a positive correlation of
400 temperature and gene expression, except for *efa* in the AU7 population (Fig. 1A).
401 ---Table 4---
402 Since in all genes, significant differences in gene expression due to increased temperature
403 were found, Tukey's honest significance test was performed in order to find out at which
404 temperatures exactly differential expression took place. There was a significant difference in
405 gene expression of both heat shock protein genes *hsp88* and *hsp98* (Fig. 1B, Fig. 1C) with
406 every temperature increase (Tukey's test: $p < 0.002$), being highly significant (Tukey's test: p
407 < 0.0001) between the 4 °C and 25 °C temperature treatments (Table 5).
408 ---Table 5---
409 The polyketide synthase genes *rPKS1, nrPKS3* and *nrPKS3'* (Fig. 1D, Fig. 1E, Fig. 1F) were
410 upregulated at the temperature increase from 4°C to 15°C as well as at 4 °C vs. 25°C (Tukey's
411 test: $p < 0.008$), but did not show a significant difference at 25 °C compared to 15 °C (Tukey's
412 test: $p > 0.06$; Table 5). In *efa*, significant upregulation was only found at 25°C compared to 4
413 °C ($p < 0.03$, see Table 5). For *efa*, *hsp98* and *rPKS1*, there was differential expression not
414 only between temperatures, but also between sites (ANOVA: $p < 0.02$, Table 4). For
415 *nrPKS3'*, a significant interaction between temperature and site was observed (ANOVA: p =

0.0115; Table 4). In AU7, an upregulation of *nrPKS3'* took place at 15 °C compared to 4 °C (Tukey's test: $p = 0.0050$) and at 25 °C compared to 4 °C (Tukey's test: $p = 0.0007$; Fig. 1; Table 6). In ST7, however, there was already a high expression of *nrPKS3'* at 4 °C, which did not increase compared to the 15 °C temperature treatment (Tukey's test: $p = 1$), while there was an upregulation at 25 °C, this upregulation was only near significant in Tukey's test ($p < 0.1$; Table 6).

---Table 6---

Genetic distance among samples of *L. pulmonaria*

Analysis of microsatellites indicated that both the Austrian and the Spanish population of *L. pulmonaria* were genetically diverse, with Austrian samples clustering together in the unrooted BIONJ tree (Fig. 2).

---Figure 2 ---

Partitioning of variance in gene expression data

Using redundancy analysis, 59.7% of the variance in gene expression were explained by regulatory (temperature), acclimatory (site), genetic, and biotic (*Plectocarpon*-infection) effects. A total of 40.3 % of the total variance was unexplained. Regulatory effects were the most important, with variation in gene expression due to temperature increase explaining 81.4 % of the explained variance (site = 2.9 %; *Plectocarpon*-infection = 0.5 %; Fig. 3). A total of 11.8 % of the explained variance was attributed to genetic factors. Covariance among variable sets amounted to 3.4 % of the explained variance. In other words, temperature treatment explained 7 times more variance than genetic distance, 28 times more variance than acclimatation to collecting site, and 156 times more variance than *Plectocarpon*-infection.

--- Figure 3 ---

Discussion

Expression stability of reference genes

Our study provides two new reference genes for qPCR studies of *Lobaria pulmonaria*. The genes *bet* and *gpd* were stable in their expression and did not vary with temperature, hence fulfilling the criteria to be used as reference genes (Bustin et al. 2009).

Effects of *Plectocarpon lichenum* infection

The overall effect of *Plectocarpon lichenum* infection on variance in gene expression was low. However, the heat shock protein gene *hsp98* showed significant infection-related differential expression in *L. pulmonaria*. Pathogen attack is known to induce upregulation of heat shock responses in plants (Aranda *et al.* 1996; Havelda and Maule 2000; Chivasa *et al.* 2005; Andrási *et al.* 2021). It is poorly known how fungi including lichenized species respond to pathogen attack, but they seem to possess the genetic mechanisms to detect and respond to pathogens (Uehling *et al.* 2017).

Effects of temperature and collecting site

The main hypothesis in our study was confirmed, i.e. thermal stress influences the expression of candidate genes for stress response. Playing an important role in refolding of denatured proteins (Miot et al. 2011; Li and Buchner 2013), most heat shock protein genes are upregulated at least in the first response to thermal stress (Plesofsky-Vig and Brambl 1998; Che et al. 2013; Park et al. 2015; Steinhäuser et al. 2016). The heat shock protein genes of the lichen-forming fungus *Lobaria pulmonaria* were indeed significantly upregulated after the temperature increases: a heat-shock response took place. Simultaneously with the heat-shock response, the PKS genes showed a significant upregulation with every temperature increase. As stress-induced polyketide production has been observed in bacteria (Auckloo *et al.* 2017)

464 and in lichen-forming fungi (Armaleo et al. 2008; Timsina et al. 2013), we had anticipated an
465 upregulation of PKS genes. Little is known about under which conditions fungal PKS genes
466 are upregulated or by which biosynthetic genes they are produced (Kim *et al.* 2021), but it has
467 been emphasized how important these compounds are for lichen tolerance of stressful biotic
468 or abiotic conditions (Huneck 1999).

469 Interestingly, elongation factor 1-α (*efa*) showed upregulation with each temperature
470 increase in *L. pulmonaria*. This gene is involved in protein biosynthesis, and specifically in
471 chain elongation by recruiting t-RNAs to ribosomes (Anand *et al.* 2003). While this gene has
472 been used as reference gene for qPCR because of its stable expression e.g. in potato (Nicot *et*
473 *al.* 2005) and cod (Aursnes *et al.* 2011), there is evidence that it is heat-induced in plants
474 (Nikolaou *et al.* 2009; Momčilović *et al.* 2016; Sun *et al.* 2020), where it may also function as
475 a molecular chaperone involved in protein degradation (Talapatra *et al.* 2002; Shin et al.
476 2009). Under higher temperatures, this gene may therefore be upregulated in lichenized fungi,
477 presumably to also function as a molecular chaperone.

478 We found a heat shock response in *L. pulmonaria* even at moderate temperatures, i.e.
479 15°C and 25°C; there was an upregulation of both *hsp88* and *hsp98* with every temperature
480 increase. In its natural growth habitat, *L. pulmonaria* is wet and physiologically active mostly
481 at temperatures up to 15 °C (Pannewitz *et al.* 2003). Apparently, moderate temperatures can
482 already provoke heat shock reactions in cold-adapted *L. pulmonaria*, although the effect was
483 much less pronounced at 15 °C than at 25 °C. Others have found a temperature of 25°C to be
484 sufficient to induce severe stress conditions in *Peltigera scabrosa* (MacFarlane and Kershaw
485 1980). The fungal gene *hsp88*, encoding a heat shock protein similar to the *hsp110* family
486 (Plesofsky-Vig and Brambl 1998), was strongly induced at 25 °C in AU7. Although the
487 expression was distinctly higher and there was no overlap among standard errors, the
488 difference between the sites was not statistically significant. This might be caused by the high

489 variance due to the small sample size of AU7. The gene *hsp98*, which encodes a prominent
490 heat shock protein (Vassilev *et al.* 1992), showed less upregulation, although there was a
491 significant difference between sites, mainly with the 15 °C treatment in AU7 showing higher
492 gene expression. This might indicate that individuals from Austria are more sensitive to heat
493 stress than those from Tenerife.
494 Response to high temperature may potentially affect many physiological processes,
495 including growth and resistance to pathogens. For example, in plants, increased temperatures
496 lead to suppressed immunity to pathogens, as higher temperature can shift the allocation of
497 heat shock proteins from defense responses to heat stress responses (Lee *et al.* 2012; Dangi *et*
498 *al.* 2018; Janda *et al.* 2019). It is conceivable that heat-stressed lichens possess a lower ability
499 to defend themselves against pathogens for the same reason. A temperature-dependent
500 reduced defense could potentially modify interactions with lichenicolous fungi, making them
501 increasingly more antagonistic. Moreover, also defense mechanisms against herbivores could
502 become weakened, which could lead to decreased survival rates.
503 Timsina *et al.* (2013) reported an increase of lichen polyketide content in *Ramalina*
504 *dilacerata* under stressful conditions and in general, polyketide content of lichens is thought
505 to confer increased tolerance to biotic and abiotic stressors (Huneck 1999). In the PKS genes
506 included in this study, expression increased significantly with the temperature rise from 4 °C
507 to 15 °C as well as highly significantly from 4 °C to 25 °C. While these results are promising,
508 more work is needed to characterize the functions of PKS genes in lichens and the pathways
509 producing specific secondary compounds (Kim et al. 2021).
510 Our data exhibited a small effect of collecting site, which represents the remaining
511 effect physiological long-term acclimatization to sites after laboratory acclimation. This
512 finding is consistent with the results of Steinhäuser *et al.* (2016), who also found collecting-
513 site related differential expression in *Peltigera membranacea* after three weeks of cold

514 acclimation to the laboratory. Collecting-site related different physiological responses to heat
515 stress were also found in *Peltigera canina* (MacFarlane and Kershaw 1980). Our two
516 collecting sites are situated in different climatic zones where the local environmental
517 conditions should be rather different (Pannewitz et al. 2003).

518 We found a significantly stronger induction of *rPKS1* in individuals from Austria when
519 compared with the Canary Islands, which, together with the stronger induced heat shock
520 protein gene expression in Austria, indicates that the gene response can vary in magnitude
521 between populations. Profound gene expression differences between populations were also
522 reported for *Peltigera membranacea* exposed to increases in temperature (Steinhäuser et al.
523 2016). In our study of *L. pulmonaria*, the residual acclimatory effects were nevertheless
524 small, explaining only 2.9 % of the explained variance, which is not surprising given that the
525 thalli were acclimated to cold during three weeks, since lichens can acclimate their
526 photosynthesis to changed conditions within a few days (Kershaw 1977; MacKenzie et al.
527 2004).

528 Expectedly, the variance in gene expression of *L. pulmonaria* in response to thermal
529 stress appeared to be mainly due to the variable we influenced in our laboratory experiment,
530 temperature; thus, the response reflects mostly an adjustment to thermal stress to maintain
531 cellular functions. That this regulatory component of variation dominates in gene expression
532 variation is perhaps not overtly surprising in a mutualistic lichen symbiosis, where a fine-
533 tuned physiological equilibrium between mycobiont and photobiont must be maintained to
534 ensure the long-term persistence of the association. Our finding that genetic differences
535 represent, with a total of 11.8 % of explained variance, the second largest component of gene
536 expression variation in response to thermal stress in *L. pulmonaria* is remarkable because it
537 implies that the three weeks' acclimation treatment to 4 °C removed most differences in gene
538 expression due to long-term physiological acclimatization to the sites of origin in Austria and

539 Tenerife – if any larger acclimatory differences existed in the first place. In our study, we did
540 not quantify the maximum (initial) acclimative effect, as our first sample was taken after
541 several weeks of acclimation to cold conditions in the lab. Other studies have found seasonal
542 light acclimation of photosynthesis in *L. pulmonaria* (Schofield *et al.* 2003) which occur via
543 macromolecular allocation to chlorophyll and RUBISCO protein (MacKenzie et al. 2004),
544 and such acclimation to changes in ambient light and temperature can occur immediately in
545 lichens, over as little time as two days (Kershaw 1977; Kershaw 1985; MacKenzie et al.
546 2004). Within the three weeks laboratory acclimation period, the samples should therefore
547 have become completely acclimated to cold.

548 As much as 40.3 % of the total variance in gene expression data was not explained by
549 the factors covered in our study. This finding is not surprising, given that gene expression
550 data tend to have a large stochastic component even for populations of clonal cells under
551 standardized conditions (McAdams and Arkin 1997; Elowitz *et al.* 2002; Blake *et al.* 2003;
552 Kærn *et al.* 2005). Much greater variance would be expected for data gathered from natural
553 populations where individuals may deviate in genomic background, physiological
554 acclimatization, phenotype, age, reproductive state, and other factors. Differences among
555 individuals might explain some of the unexplained variation in gene expression. Substantial
556 inter-individual variation in gene expression has also been reported for another Peltigeralean
557 lichen, *Peltigera membranacea* (Steinhäuser et al. 2016).

558

559 # Conclusions

560 The lichen-forming fungus *Lobaria pulmonaria* may provide an interesting model for *in*
561 *vivo* studies of heat shock responses. Overall, our results show clearly that gene expression
562 variation in *L. pulmonaria* under thermal stress is substantially influenced by the abiotic
563 environment (temperature), with regulatory effects predominating, i.e. direct responses to

elevated temperature. Lichen-forming fungi have evolved powerful molecular pathways to withstand environmental fluctuations and stress, and heat shock responses are a critical component conveying stress tolerance. Our results suggest that the colonization of thalli by lichenicolous fungi might have an influence on the mycobiont's heat shock responses; abiotic and biotic factors appear to cause cumulative effects. While *L. pulmonaria* has the molecular machinery to counteract short-term thermal stress, its persistence in a given landscape depends on the overall long-term positive carbon balance, which can be compromised by warmer temperatures leading to increased respiration rates and by reduced precipitation during summer, and both have been predicted for Central Europe in connection with global climate change (Middelkoop *et al.* 2001; Ahrens *et al.* 2014; IPCC 2021). These topics deserve more attention in future work.

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References

Adamack AT and Gruber B (2014) PopGenReport: simplifying basic population genetic analyses in R. *Methods in Ecology and Evolution* **5**, 384-387.

Ahrens B, Formayer H, Gobiet A, Heinrich G, Hofstätter M, Matulla C, Prein AF, Truhetz H, Anders I, Haslinger K *et al.* (2014) *Zukünftige Klimaentwicklung*. In: Kromp-Kolb H, Nakicenovic N, Steininger K, Gobiet A, Formayer H, Köppl A, Prettenthaler F, Stötter J and Schneider J (eds), *Österreichischer Sachstandsbericht*

591 *Klimawandel 2014 (AAR14)*. Wien: Verlag der Österreichischen Akademie der
592 Wissenschaften, pp. 301-346.
593 **Albrecht D, Guthke R, Brakhage AA and Kniemeyer O** (2010) Integrative analysis of the
594 heat shock response in *Aspergillus fumigatus*. *BMC Genomics* **11**, 32.
595 **Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ**
596 (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search
597 programs. *Nucleic Acids Research* **25**, 3389-3402.
598 **Anand M, Chakraburtty K, Marton MJ, Hinnebusch AG and Kinzy TG** (2003)
599 Functional interactions between yeast translation eukaryotic elongation factor (eEF)
600 1A and eEF3. *Journal of Biological Chemistry* **278**, 6985-6991.
601 **Andersen CL, Ledet-Jensen J and Ørntoft T** (2004) Normalization of real-time
602 quantitative RT-PCR data: a model based variance estimation approach to identify
603 genes suited for normalization - applied to bladder- and colon-cancer data-sets.
604 *Cancer Research* **64**, 5245-5250.
605 **Andrási N, Pettkó-Szandtner A and Szabados L** (2021) Diversity of plant heat shock
606 factors: regulation, interactions, and functions. *Journal of Experimental Botany* **72**,
607 1558-1575.
608 **Aranda MA, Escaler M, Wang D and Maule AJ** (1996) Induction of HSP70 and
609 polyubiquitin expression associated with plant virus replication. *Proceedings of the*
610 *National Academy of Sciences* **93**, 15289.
611 **Armaleo D, Zhang Y and Cheung S** (2008) Light might regulate divergently depside and
612 depsidone accumulation in the lichen *Parmotrema hypotropum* by affecting thallus
613 temperature and water potential. *Mycologia* **100**, 565-576.
614 **Arshad MS, Farooq M, Asch F, Krishna JSV, Prasad PVV and Siddique KHM** (2017)
615 Thermal stress impacts reproductive development and grain yield in rice. *Plant*
616 *Physiology and Biochemistry* **115**, 57-72.
617 **Asplund J and Gauslaa Y** (2008) Mollusc grazing limits growth and early development of
618 the old forest lichen *Lobaria pulmonaria* in broadleaved deciduous forests. *Oecologia*
619 **155**, 93-99.
620 **Asplund J, Gauslaa Y and Merinero S** (2016) The role of fungal parasites in tri-trophic
621 interactions involving lichens and lichen-feeding snails. *New Phytologist* **211**, 1352-
622 1357.
623 **Asplund J, Gauslaa Y and Merinero S** (2018) Low synthesis of secondary compounds in
624 the lichen *Lobaria pulmonaria* infected by the lichenicolous fungus *Plectocarpon*
625 *lichenum*. *New Phytologist* **217**, 1397-1400.
626 **Auckloo BN, Pan C, Akhter N, Wu B, Wu X and He S** (2017) Stress-driven discovery of
627 novel cryptic antibiotics from a marine fungus *Penicillium* sp. BB1122. *Frontiers in*
628 *Microbiology* **8**, 1450.
629 **Aursnes IA, Rishovd AL, Karlsen HE and Gjøen T** (2011) Validation of reference genes
630 for quantitative RT-qPCR studies of gene expression in Atlantic cod (*Gadus morhua*
631 l.) during temperature stress. *BMC Research Notes* **4**, 104.
632 **Bergmann TC and Werth S** (2017) Intrathalline distribution of two lichenicolous fungi on
633 *Lobaria* hosts – an analysis based on quantitative Real-Time PCR. *Herzogia* **30**, 253-
634 271.
635 **Bertrand RL and Sorensen JL** (2018) A comprehensive catalogue of polyketide synthase
636 gene clusters in lichenizing fungi. *Journal of Industrial Microbiology and*
637 *Biotechnology* **45**, 1067-1081.
638 **Bidussi M, Goward T and Gauslaa Y** (2013) Growth and secondary compound investments
639 in the epiphytic lichens *Lobaria pulmonaria* and *Hypogymnia occidentalis*
640 transplanted along an altitudinal gradient in British Columbia. *Botany-Botanique* **91**,

621-630.
Blake WJ, Kærn M, Cantor CR and Collins JJ (2003) Noise in eukaryotic gene expression. *Nature* **422**, 633-637.
Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL *et al.* (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**, 611-622.
Carmo LST, Martins ACQ, Martins CCC, Passos MAS, Silva LP, Araujo ACG, Brasileiro ACM, Miller RNG, Guimarães PM and Mehta A (2019) Comparative proteomics and gene expression analysis in *Arachis duranensis* reveal stress response proteins associated to drought tolerance. *Journal of Proteomics* **192**, 299-310.
Che S, Song W and Lin X (2013) Response of heat-shock protein (HSP) genes to temperature and salinity stress in the antarctic psychrotrophic bacterium *Psychrobacter* sp. G. *Current Microbiology* **67**, 601-608.
Cheviron ZA, Whitehead A and Brumfield RT (2008) Transcriptomic variation and plasticity in rufous-collared sparrows (*Zonotrichia capensis*) along an altitudinal gradient. *Molecular Ecology* **17**, 4556-4569.
Chivasa S, Simon WJ, Yu X-L, Yalpani N and Slabas AR (2005) Pathogen elicitor-induced changes in the maize extracellular matrix proteome. *PROTEOMICS* **5**, 4894-4904.
Cornejo C, Scheidegger C and Honegger R (2015) Axenic cultivation of mycelium of the lichenized fungus, *Lobaria pulmonaria* (Peltigerales, Ascomycota). *Bio-protocol* **5**, e1513.
Dangi AK, Sharma B, Khangwal I and Shukla P (2018) Combinatorial interactions of biotic and abiotic stresses in plants and their molecular mechanisms: Systems biology approach. *Mol Biotechnol* **60**, 636-650.
Dar TUH, Dar SA, Islam SU, Mangral ZA, Dar R, Singh BP, Verma P and Haque S (2021) Lichens as a repository of bioactive compounds: an open window for green therapy against diverse cancers. *Seminars in Cancer Biology* **In Press**.
de Nadal E, Ammerer G and Posas F (2011) Controlling gene expression in response to stress. *Nature Reviews Genetics* **12**, 833-845.
De Wit P, Pespeni MH, Ladner JT, Barshis DJ, Seneca F, Jaris H, Therkildsen NO, Morikawa M and Palumbi SR (2012) The simple fool's guide to population genomics via RNA-Seq: an introduction to high-throughput sequencing data analysis. *Molecular Ecology Resources* **12**, 1058-1067.
Dixon G, Abbott E and Matz M (2020) Meta-analysis of the coral environmental stress response: *Acropora* corals show opposing responses depending on stress intensity. *Molecular Ecology* **29**, 2855-2870.
Doering JA, Miao VPW and Piercey-Normore MD (2014) Rehydration conditions for isolation of high quality RNA from the lichen *Lobaria pulmonaria*. *BMC Research Notes* **7**, 442-442.
Elowitz MB, Levine AJ, Siggia ED and Swain PS (2002) Stochastic gene expression in a single cell. *Science* **297**, 1183-1186.
Evans TG, Chan F, Menge BA and Hofmann GE (2013) Transcriptomic responses to ocean acidification in larval sea urchins from a naturally variable pH environment. *Molecular Ecology* **22**, 1609-1625.
Franzmann TM, Menhorn P, Walter S and Buchner J (2008) Activation of the chaperone Hsp26 is controlled by the rearrangement of its thermosensor domain. *Molecular Cell* **29**, 207-216.
Gasch AP (2007) Comparative genomics of the environmental stress response in ascomycete

691 fungi. *Yeast* **24**, 961-976.
692 **Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D and**
693 **Brown PO** (2000) Genomic expression programs in the response of yeast cells to
694 environmental changes. *Molecular Biology of the Cell* **11**, 4241-4257.
695 **Gascuel O** (1997) BIONJ: an improved version of the NJ algorithm based on a simple model
696 of sequence data. *Molecular Biology and Evolution* **14**, 685-695.
697 **Gauslaa Y and McEvoy M** (2005) Seasonal changes in solar radiation drive acclimation of
698 the sun-screening compound parietin in the lichen *Xanthoria parietina*. *Basic and*
699 *Applied Ecology* **6**, 75-82.
700 **Gauslaa Y, Bidussi M, Solhaug KA, Asplund J and Larsson P** (2013) Seasonal and spatial
701 variation in carbon based secondary compounds in green algal and cyanobacterial
702 members of the epiphytic lichen genus *Lobaria*. *Phytochemistry* **94**, 91-98.
703 **Hagiwara D, Sakamoto K, Abe K and Gomi K** (2016) Signaling pathways for stress
704 responses and adaptation in *Aspergillus* species: stress biology in the post-genomic
705 era. *Biosci Biotechnol Biochem* **80**, 1667-1680.
706 **Hamann E, Kesselring H, Armbruster GFJ, Scheepens JF and Stöcklin J** (2016)
707 Evidence of local adaptation to fine- and coarse-grained environmental variability in
708 *Poa alpina* in the Swiss Alps. *Journal of Ecology* **104**, 1627-1637.
709 **Havelda Z and Maule AJ** (2000) Complex spatial responses to cucumber mosaic virus
710 infection in susceptible *Cucurbita pepo* cotyledons. *The Plant Cell* **12**, 1975-1986.
711 **Huneck S** (1999) The significance of lichens and their metabolites. *Naturwissenschaften* **86**,
712 559-570.
713 **IPCC** (2021) *Climate Change 2021: The physical science basis. Contribution of Working*
714 *Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate*
715 *Change*. In: Masson-Delmotte V, Zhai P, Pirani A, Connors SL, Péan C, Berger S,
716 Caud N, Chen Y, Goldfarb L, Gomis MI *et al.* (eds), vol. In Press. Cambridge:
717 Cambridge University Press.
718 **Jamil A, Riaz S, Ashraf M and Foolad MR** (2011) Gene expression profiling of plants
719 under salt stress. *Critical Reviews in Plant Sciences* **30**, 435-458.
720 **Janda M, Lamparová L, Zubíková A, Burketová L, Martinec J and Krčková Z** (2019)
721 Temporary heat stress suppresses PAMP-triggered immunity and resistance to bacteria
722 in Arabidopsis thaliana. *Molecular Plant Pathology* **20**, 1005-1012.
723 **Joneson S, Armaleo D and Lutzoni F** (2011) Fungal and algal gene expression in early
724 developmental stages of lichen-symbiosis. *Mycologia* **103**, 291-306.
725 **Kærn M, Elston TC, Blake WJ and Collins JJ** (2005) Stochasticity in gene expression:
726 From theories to phenotypes. *Nature Reviews Genetics* **6**, 451-464.
727 **Kershaw AP** (1985) *Physiological ecology of lichens*. Cambridge: Cambridge University
728 Press.
729 **Kershaw KA** (1977) Physiological-environmental interactions in lichens. III. The rate of net
730 photosynthetic acclimation in *Peltigera canina* (L.) Willd var. *praetextata* (Floerke in
731 Somm.) Hue, and *P. polydactyla* (Neck.) Hoffm. *New Phytologist* **79**, 391-402.
732 **Khosla C, Gokhale RS, Jacobsen JR and Cane DE** (1999) Tolerance and specificity of
733 polyketide synthases. *Annual Review of Biochemistry* **68**, 219-253.
734 **Kim W, Jeong M-H, Yun S-H and Hur J-S** (2021) Transcriptome analysis identifies a gene
735 cluster for the biosynthesis of biruloquinone, a rare phenanthraquinone, in a lichen-
736 forming fungus *Cladonia macilenta*. *Journal of Fungi* **7**.
737 **Kosman E and Leonard KJ** (2005) Similarity coefficients for molecular markers in studies
738 of genetic relationships between individuals for haploid, diploid, and polyploid
739 species. *Molecular Ecology* **14**, 415-424.
740 **Lawrey JD** (1986) Biological role of lichen substances. *Bryologist* **89**, 111-122.

Lawrey JD (1989) Lichen secondary compounds: evidence for a correspondence between antiherbivore and antimicrobial function. *The Bryologist* **92**, 326-328.
Lawrey JD (2000) Chemical interactions between two lichen-degrading fungi. *Journal of Chemical Ecology* **26**, 1821-1831.
Lawrey JD and Diederich P (2003) Lichenicolous fungi: Interactions, evolution, and biodiversity. *Bryologist* **106**, 80-120.
Lee JH, Yun HS and Kwon C (2012) Molecular communications between plant heat shock responses and disease resistance. *Molecules and Cells* **34**, 109-116.
Li J and Buchner J (2013) Structure, function and regulation of the Hsp90 machinery. *Biomedical Journal* **36**, 106-117.
MacFarlane JD and Kershaw KA (1980) Physiological-environmental interactions in lichens. IX. Thermal stress and lichen ecology. *New Phytologist* **84**, 669-685.
MacKenzie TDB, Johnson J and Campbell DA (2004) Environmental change provokes rapid macromolecular reallocations within the photosynthetic system in a static population of photobionts in the lichen *Lobaria pulmonaria*. *Lichenologist* **36**, 425-433.
MacKenzie TDB, MacDonald TM, Dubois LA and Campbell DA (2001) Seasonal changes in temperature and light drive acclimation of photosynthetic physiology and macromolecular content in *Lobaria pulmonaria*. *Planta* **214**, 57-66.
McAdams HH and Arkin A (1997) Stochastic mechanisms in gene expression. *Proceedings of the National Academy of Science of the United States of America* **94**, 814-819.
Merinero S, Bidussi M and Gauslaa Y (2015) Do lichen secondary compounds play a role in highly specific fungal parasitism? *Fungal Ecology* **14**, 125–129.
Miao VPW, Manoharan SS, Snæbjarnarson V and Andrésson ÓS (2012) Expression of lec-1, a mycobiont gene encoding a galectin-like protein in the lichen *Peltigera membranacea*. *Symbiosis* **57**, 23-31.
Middelkoop H, Daamen K, Gellens D, Grabs W, Kwadijk JCJ, Lang H, Parmet B, Schadler B, Schulla J and Wilke K (2001) Impact of climate change on hydrological regimes and water resources management in the Rhine basin. *Climatic Change* **49**, 105-128.
Miot M, Reidy M, Doyle SM, Hoskins JR, Johnston DM, Genest O, Vitery MC, Masison DC and Wickner S (2011) Species-specific collaboration of heat shock proteins (Hsp) 70 and 100 in thermotolerance and protein disaggregation. *Proceedings of the National Academy of Science of the United States of America* **108**, 6915-6920.
Mizoguchi T, Ichimura K and Shinozaki K (1997) Environmental stress response in plants: the role of mitogen-activated protein kinases. *Trends in Biotechnology* **15**, 15-19.
Momčilović I, Pantelić D, Zdravković-Korać S, Oljača J, Rudić J and Fu J (2016) Heat-induced accumulation of protein synthesis elongation factor 1A implies an important role in heat tolerance in potato. *Planta* **244**, 671-679.
Nicot N, Hausman J-F, Hoffmann L and Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *Journal of Experimental Botany* **56**, 2907-2914.
Nikolaou E, Agrafioti I, Stumpf M, Quinn J, Stansfield I and Brown AJP (2009) Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evolutionary Biology* **9**, 44.
Nivina A, Yuet KP, Hsu J and Khosla C (2019) Evolution and diversity of assembly-line polyketide synthases. *Chemical Reviews* **119**, 12524-12547.
Nybakken L, Helmersen AM, Gauslaa Y and Selas V (2010) Lichen compounds restrain lichen feeding by bank voles (*Myodes glareolus*). *Journal of Chemical Ecology* **36**, 298-304.

O'Meara TR, O'Meara MJ, Polvi EJ, Pourhaghighi MR, Liston SD, Lin Z-Y, Veri AO, Emili A, Gingras A-C and Cowen LE (2019) Global proteomic analyses define an environmentally contingent Hsp90 interactome and reveal chaperone-dependent regulation of stress granule proteins and the R2TP complex in a fungal pathogen. *Plos Biology* **17**, e3000358.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH and Wagner HH (2016) vegan: Community Ecology Package. R package version 2.3-3. URL: http://CRAN.R-project.org/package=vegan.

Palumbi SR, Barshis DJ, Traylor-Knowles N and Bay RA (2014) Mechanisms of reef coral resistance to future climate change. *Science* **344**, 895.

Pannewitz S, Schroeter B, Scheidegger C and Kappen L (2003) Habitat selection and light conditions: a field study with *Lobaria pulmonaria*. *Bibliotheca Lichenologica* **86**, 281-297.

Paradis E (2006) *Analysis of phylogenetics and evolution with R*. New York, United States: Springer.

Paradis E, Claude J and Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289-290.

Park K, Lee JS, Kang J-C, Kim JW and Kwak I-S (2015) Cascading effects from survival to physiological activities, and gene expression of heat shock protein 90 on the abalone *Haliotis discus hannai* responding to continuous thermal stress. *Fish & Shellfish Immunology* **42**, 233-240.

Park NS, Kim YG, Kim KK, Park HC, Son HJ, Hong CH and Lee SM (2014) Molecular cloning of the cDNA of heat shock protein 88 gene from the entomopathogenic fungus, *Paecilomyces tenuipes* Jocheon-1. *International Journal of Industrial Entomology* **28**, 71-84.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, e45.

Plesofsky-Vig N and Brambl R (1998) Characterization of an 88-kDa heat shock protein of *Neurospora crassa* that interacts with Hsp30. *The Journal of Biological Chemistry* **273**, 11335–11341.

R Core Team (2018) *R: A language and environment for statistical computing (version 3.6.3)*. Vienna, Austria: R Foundation for Statistical Computing.

Ranković B and Kosanić M (2019) *Lichens as a potential source of bioactive secondary metabolites*. In: Ranković B (ed), *Lichen Secondary Metabolites: Bioactive Properties and Pharmaceutical Potential*. Cham: Springer International Publishing, pp. 1-29.

Roncarati D and Scarlato V (2017) Regulation of heat-shock genes in bacteria: from signal sensing to gene expression output. *FEMS Microbiology Reviews* **41**, 549-574.

Scheidegger C, Bilovitz PO, Werth S, Widmer I and Mayrhofer H (2012) Hitchhiking with forests: population genetics of the epiphytic lichen *Lobaria pulmonaria* in primeval and managed forests in Southeastern Europe. *Ecology and Evolution* **2**, 2223-2240.

Schipperges B, Kappen L and Sonesson M (1995) Intraspecific variations of morphology and physiology of temperate to arctic populations of *Cetraria nivalis*. *Lichenologist* **27**, 517-529.

Schofield SC, Campbell DA, Funk C and MacKenzie TDB (2003) Changes in macromolecular allocation in nondividing algal symbionts allow for photosynthetic acclimation in the lichen *Lobaria pulmonaria*. *New Phytologist* **159**, 709-718.

Shin D, Moon S-J, Park SR, Kim B-G and Byun M-O (2009) Elongation factor 1α from *A. thaliana* functions as molecular chaperone and confers resistance to salt stress in yeast and plants. *Plant Science* **177**, 156-160.

Shrestha G and St. Clair LL (2013) Lichens: a promising source of antibiotic and anticancer drugs. *Phytochemistry Reviews* **12**, 229-244.
Smith HA, Burns AR, Shearer TL and Snell TW (2012) Three heat shock proteins are essential for rotifer thermotolerance. *Journal of Experimental Marine Biology and Ecology* **413**, 1-6.
Steinhäuser SS, Andrésson ÓS, Pálsson A and Werth S (2016) Fungal and cyanobacterial gene expression in a lichen symbiosis: Effect of temperature and location. *Fungal Biology* **120**, 1194–1208.
Suleyman H, Odabasoglu F, Aslan A, Cakir A, Karagoz Y, Gocer F, Halici M and Bayir Y (2003) Anti-inflammatory and antiulcerogenic effects of the aqueous extract of *Lobaria pulmonaria* (L.) Hoffm. *Phytomedicine* **10**, 552-557.
Sun D, Ji X, Jia Y, Huo D, Si S, Zeng L, Zhang Y and Niu L (2020) LreEF1A4, a translation elongation factor from *Lilium regale,* is pivotal for cucumber mosaic virus and tobacco rattle virus infections and tolerance to salt and drought. *International Journal of Molecular Sciences* **21**.
Takahashi H, Kusuya Y, Hagiwara D, Takahashi-Nakaguchi A, Sakai K and Gonoi T (2017) Global gene expression reveals stress-responsive genes in *Aspergillus fumigatus* mycelia. *BMC Genomics* **18**, 942-942.
Talapatra S, Wagner JDO and Thompson CB (2002) Elongation factor-1 alpha is a selective regulator of growth factor withdrawal and ER stress-induced apoptosis. *Cell Death & Differentiation* **9**, 856-861.
Terhorst A, Sandikci A, Keller A, Whittaker CA, Dunham MJ and Amon A (2020) The environmental stress response causes ribosome loss in aneuploid yeast cells. *Proceedings of the National Academy of Sciences* **117**, 17031.
Timsina BA, Sorensen JL, Weihrauch D and Piercey-Normore MD (2013) Effect of aposymbiotic conditions on colony growth and secondary metabolite production in the lichen-forming fungus *Ramalina dilacerata*. *Fungal Biology* **117**, 731-743.
Torzilli AP, Mikelson PA and Lawrey JD (1999) Physiological effect of lichen secondary metabolites on the lichen parasite *Marchandiomyces corallinus*. *Lichenologist* **31**, 307-314.
Uehling J, Deveau A and Paoletti M (2017) Do fungi have an innate immune response? An NLR-based comparison to plant and animal immune systems. *PLoS Pathogens* **13**, e1006578.
Vassilev AO, Plesofsky-Vig N and Brambl R (1992) Isolation, partial amino acid sequence, and cellular distribution of heat-shock protein hsp98 from *Neurospora crassa*. *Biochimica et Biophysica Acta (BBA)* **1156**, 1-6.
Wang YY, Zhang XY, Zhou QM, Zhang XL and Wei JC (2015) Comparative transcriptome analysis of the lichen-forming fungus *Endocarpon pusillum* elucidates its drought adaptation mechanisms. *Science China Life Sciences* **58**, 89-100.
Werth S, Cornejo C and Scheidegger C (2013) Characterization of microsatellite loci in the lichen fungus *Lobaria pulmonaria* (Lobariaceae). *Applications in Plant Sciences* **1**, apps. 1200290.
Whitehead A, Triant DA, Champlin D and Nacci D (2010) Comparative transcriptomics implicates mechanisms of evolved pollution tolerance in a killifish population. *Molecular Ecology* **19**, 5186-5203.
Widmer I, Dal Grande F, Cornejo C and Scheidegger C (2010) Highly variable microsatellite markers for the fungal and algal symbionts of the lichen *Lobaria pulmonaria* and challenges in developing biont-specific molecular markers for fungal associations. *Fungal Biology* **114**, 538-544.
Yang M-X, Devkota S, Wang L-S and Scheidegger C (2021) Ethnolichenology—The use

of lichens in the Himalayas and southwestern parts of China. *Diversity* **13**, 330.
Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden TL (2012) Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134.
Zhang L, Zhang X and Fan S (2017) Meta-analysis of salt-related gene expression profiles identifies common signatures of salt stress responses in *Arabidopsis*. *Plant Systematics and Evolution* **303**, 757-774.

Tables

Table 1: Reference and candidate genes used for *Lobaria pulmonaria*, including GenBank accession (Accession); gene abbreviation (Gene); gene name; Coord. LPU, alignment coordinates of blast hit on the *L. pulmonaria* genome; name of gene model from the *L. pulmonaria* Scotland JGI v1.0 reference genome; gene model name in the *L. pulmonaria* Scotland reference genome (Gene model LPU); ProteinID, protein ID associated with *L. pulmonaria* gene model;; KOG Class, KOG functional class assignment; KOG Descr., description of KOG function; KOG ID; number of exons (Nr. exons); e-value from BLASTN analysis against the *L. pulmonaria* reference genome (e-value LPU); Id. LPU, percent identity of blast hit to *L. pulmonaria* reference genome. The loci nrPKS3 and nrPKS3' are exons of the same polyketide synthase gene.

Accession	Gene	Name	Coord. LPU	Gene model LPU	ProteinID	KOG Class	KOG Descr	KOG ID	Nr. exons	e-value LPU	Id LPU
KX866403	*bet*	β-tubulin	scaffold_685:14464-15096	CE775768_21397	775769	Cytoskeleton	Beta tubulin	KOG1375	8	3,24E-99	100%
KX866404	*efa*	Elongation factor 1-α	scaffold_766:8010-9411	fgenesh1_kg.766_#_6_#_TRINITY_DN10494_c1_g1_i3	1228547	Translation, ribosomal structure and biogenesis	Translation elongation factor EF-1 alpha/Tu	KOG0052	7	0,00E+00	100%
KX866402	*gpd*	Glyceraldehyde 3-phosphate dehydrogenase	scaffold_272:44382-46782	fgenesh1_kg.272_#_46_#_TRINITY_DN11298_c8_g2_i3	1201865	Carbohydrate transport and metabolism	Glyceraldehyde 3-phosphate dehydrogenase	KOG0657	2	0,00E+00	100%
KX866400	*hsp88*	Heat shock protein *Hsp88*	scaffold_78:93452-94065	e_gw1.78.24.1	1078087	Posttranslational modification, protein turnover, chaperones	Molecular chaperones HSP105/HSP110/SSE1, HSP70 superfamily	KOG0103	5	0,00E+00	100%
KX866401	*hsp98*	Heat shock protein *Hsp98/Hsp104/ClpA*	scaffold_10:205498-206226	gm1.608_g	1258478	Posttranslational modification, protein turnover, chaperones	Chaperone HSP104 and related ATP-dependent Clp proteases	KOG1051	1	0,00E+00	100%
KX866397	*rPKS1*	Reducing type I polyketide synthase	scaffold_432:15800-16594	CE565179_9106	565180	Lipid transport and metabolism	Animal-type fatty acid synthase and related proteins	KOG1202	5	0,00E+00	100%
KX866398	*nrPKS3*	Non-reducing type I polyketide synthase	scaffold_1083:6354-7345	MIX1700_1158_6	1274420	Lipid transport and metabolism	Animal-type fatty acid synthase and related proteins	KOG1202	6	0,00E+00	99%
KX866399	*nrPKS3'*	Non-reducing type I polyketide synthase	scaffold_1083:4743-5599	MIX1700_1158_6	1274420	Lipid transport and metabolism	Animal-type fatty acid synthase and related proteins	KOG1202	6	0,00E+00	100%

Table 2: Reference and candidate genes for *Lobaria pulmonaria*, showing gene names, forward and reverse primer sequences and primer efficiency (Eff.).

Gene ID	Forward primer 5‘-3‘	Reverse primer 5‘-3‘	Eff. [%]
gpd	TCCAACGCCTCATGTACGAC	GTGCTGCTGGGGATGATGTT	93.9
bet	CAATTCGGCACCCTCGGT	ACAACAAATATGTGCCTCGTGC	93.4
efa	TGAATCCGACGTTGTCACCC	AAAGCCCTCCGTCTTCCTCT	92.1
hsp88	CTCTGAACCAGGATGAAGCCG	GAATGGCTGCTTGCGGTAGA	90.7
hsp98	GACGCCAGGTTCTCCAATCA	AGTAGACTCGAAGACTGCCGA	88.0
rPKS1	GTTGTTCTTGGCTCCGCAAC	CGCACAAACACGTCGGTAAC	92.0
nrPKS3	TTGGGCTGAAGATTGCGACA	CTCGGCATCCTCAAGACGTT	91.6
nrPKS3’	CAAGAGACTGTCCTGAGCGG	AAGTGGGGAGATCACCGGAA	92.4

Table 3: *p*-values of Student's *t*-test for the differences in gene expression between individuals of the ST7 population with and without *Plectocarpon lichenum* infection at 4 °C, 15 °C and 25 °C.

Gene	**4 °C**	**15 °C**	**25 °C**
efa	0.4084	0.8715	0.5991
hsp88	0.6969	0.9907	0.7800
hsp98	0.7305	0.4527	**0.0102**
rPKS1	0.2036	0.2184	0.7434
nrPKS3	0.2289	0.6189	0.6221
nrPKS3'	0.6934	0.1095	0.2802

Table 4: *p*-values of ANOVA, using a linear mixed effects model with temperature and habitat as fixed factors and site and lichen individual as random factors, for differences in the expression of the heat shock protein genes, *hsp88* and *hsp98*, elongation factor 1-α *efa* and the polyketide synthase genes *rPKS1*, *nrPKS3* and *nrPKS3'*.

Gene	Temperature	Site	Interaction
efa	**0.0084**	**0.0138**	0.1851
hsp88	**<0.0001**	0.0526	0.2701
hsp98	**<0.0001**	**0.0009**	0.4198
rPKS1	**<0.0001**	**0.0129**	0.0744
nrPKS3	**0.0009**	0.1885	0.5579
nrPKS3'	**0.0001**	0.1619	**0.0115**

Table 5: *p*-values of Tukey's honest significance test for differences in the expression of the heat shock protein genes *hsp88* and *hsp98,* the elongation factor 1-α *efa* and the polyketide synthase genes *rPKS1*, *nrPKS3* and *nrPKS3'* due to temperature treatments at 4 °C, 15 °C and 25 °C.

Gene ID	**4 vs. 15 °C**	**15 vs. 25 °C**	**4 vs. 25 °C**
efa	0.1979	0.5356	**0.0221**
hsp88	**0.0011**	**<0.0001**	**<0.0001**
hsp98	**0.0007**	**0.0002**	**<0.0001**
rPKS1	**0.0010**	0.0674	**<0.0001**
nrPKS3	**0.0079**	0.7271	**0.0012**
nrPKS3'	**0.0057**	0.0861	**<0.0001**

Table 6: *p*-values of Tukey's honest significance test for differences in the expression of the polyketide synthase gene nrPKS3' due to the temperature treatments at 4 °C, 15 °C and 25 °C in individuals from the sites AU7 and ST7 and both sites combined.

Site	**4 vs. 15 °C**	**15 vs. 25 °C**	**4 vs. 25 °C**
AU7	**0.0050**	0.9725	**0.0007**
ST7	1.0000	0.0929	0.0613
Combined	**0.0001**	0.1619	**0.0115**

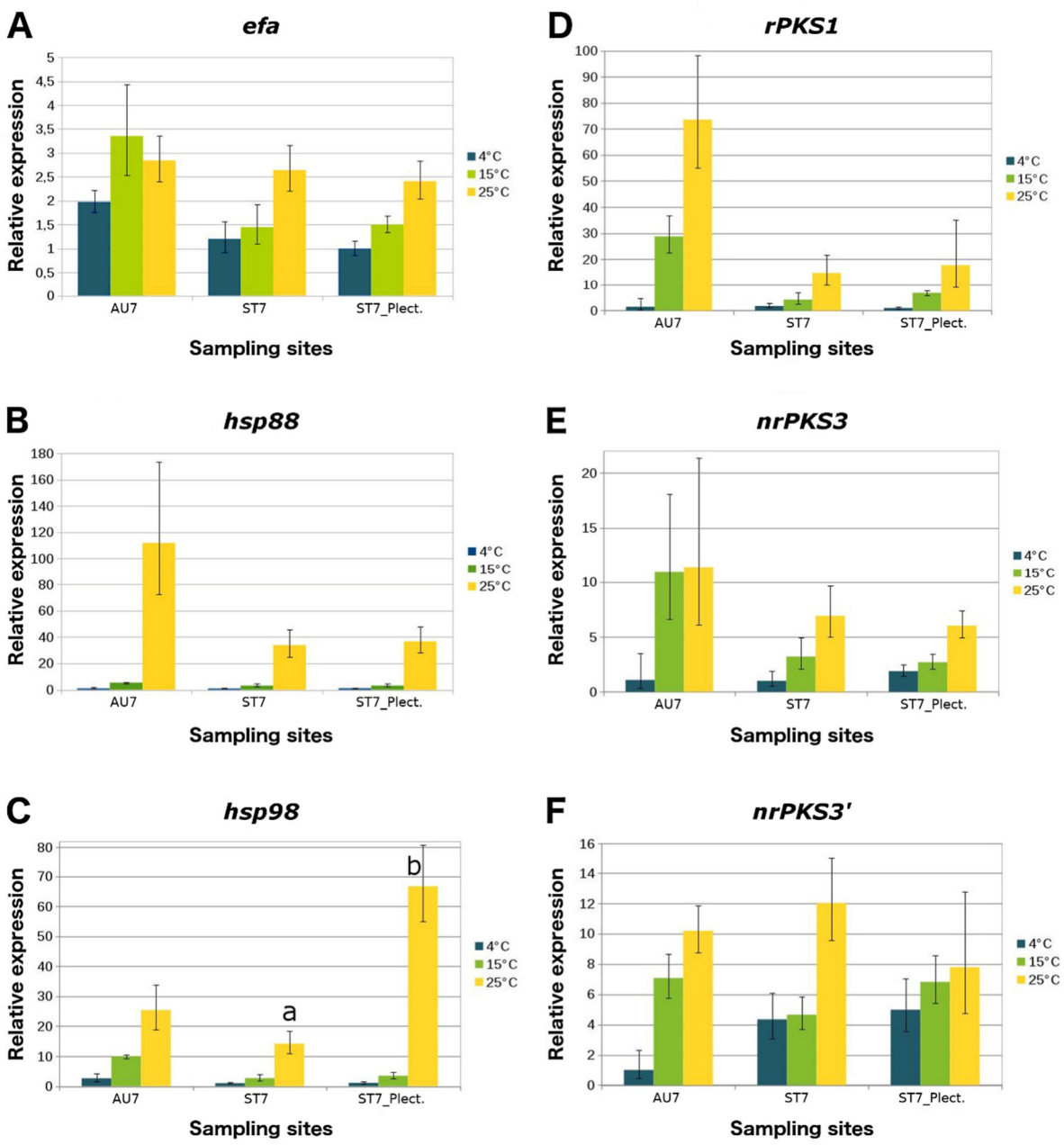

Figure 1: Relative expression of mycobiont genes in thalli of the epiphytic lichen *Lobaria pulmonaria* from collecting sites AU7 (Austria) and ST7 (Spain, Tenerife) at 4 °C, 15 °C and 25 °C. For ST7, thalli with and without stromata of the lichenicolous fungus *Plectocarpon lichenum* were compared. The thallus with lowest expression was set to one. The loci *nrPKS3* and *nrPKS3'* represent two exons of the same gene. The letters "a" and "b" indicate a significant expression difference between samples infected with *P. lichenum* and those without.

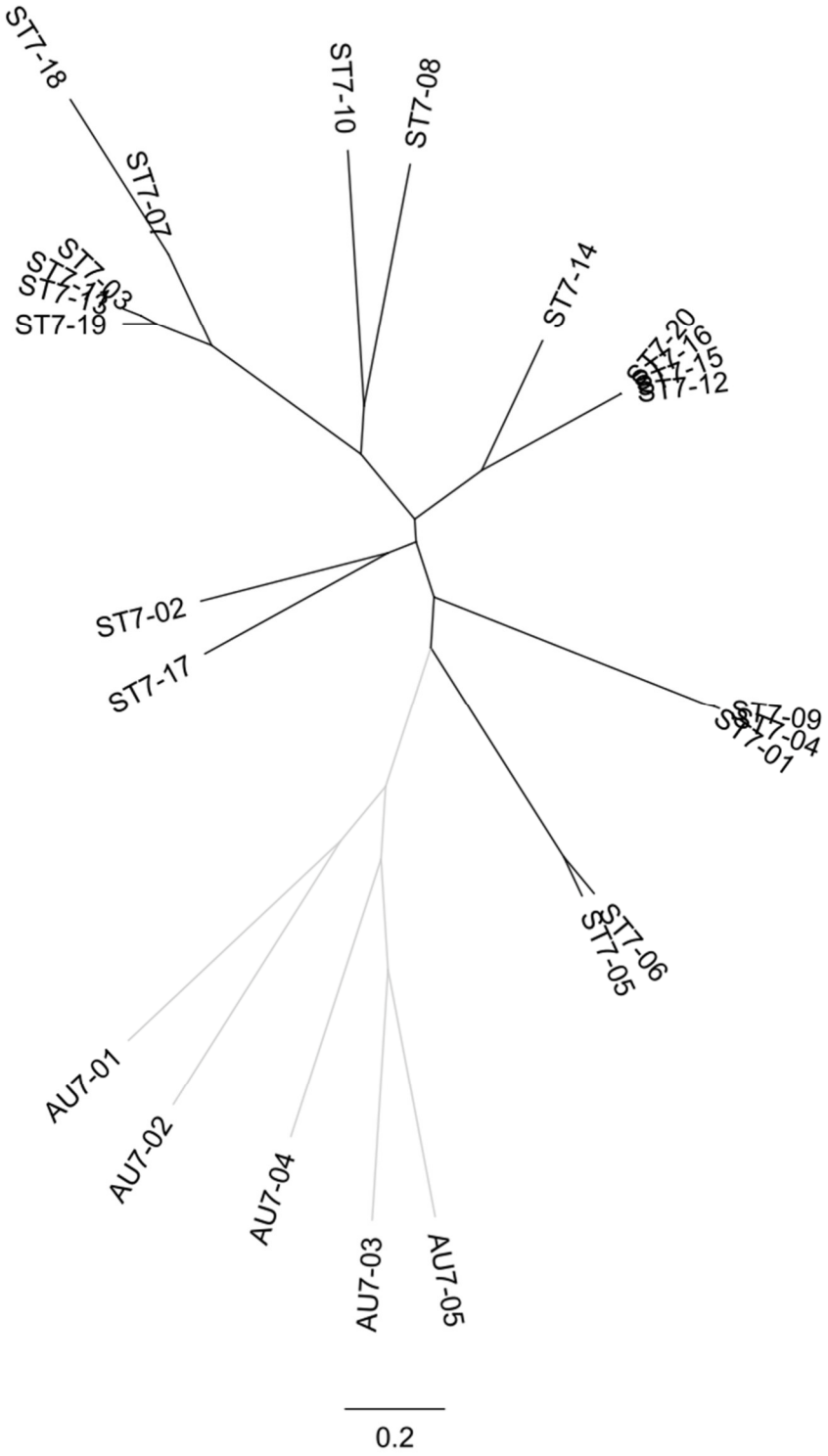

Figure 2: Unrooted BIONJ neighbor-joining tree for 11 microsatellite loci of the 25 *Lobaria pulmonaria* samples from Austria (AU7) and Spain (ST7) included in the gene expression experiment. Branches containing Austrian samples are shown in grey.

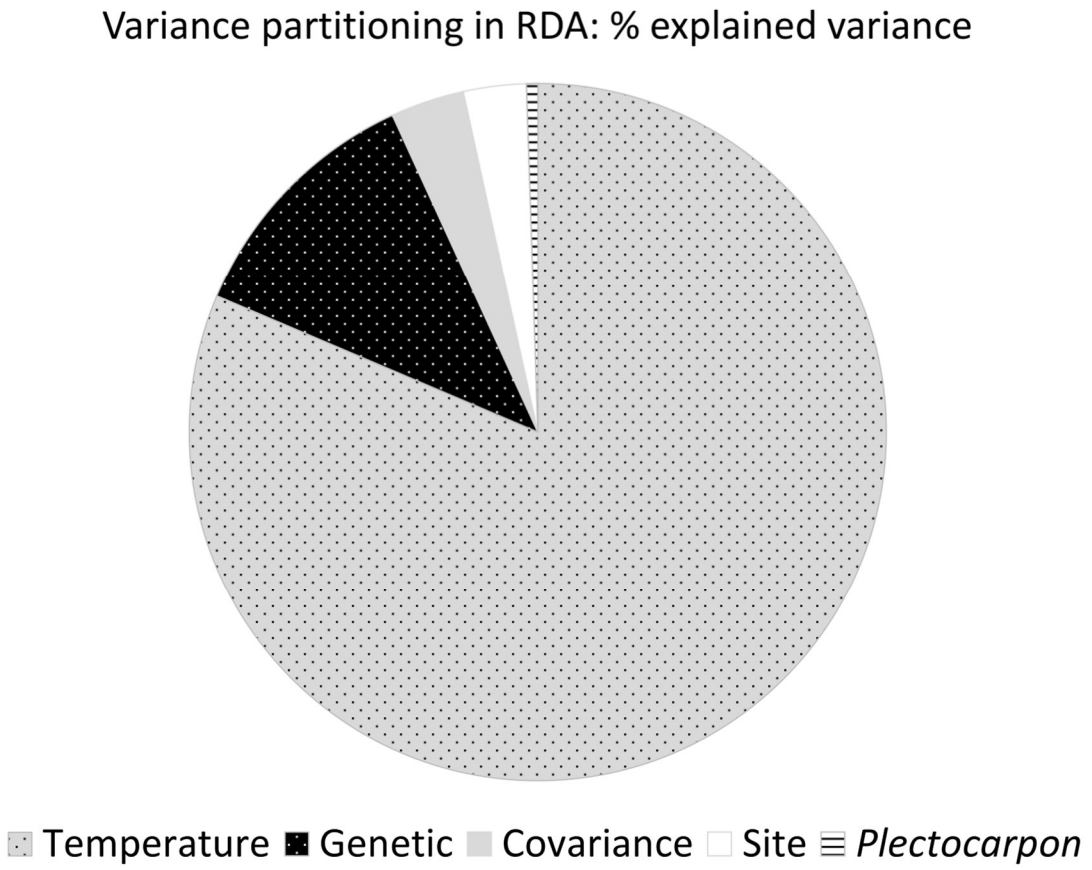

Figure 3. Partitioning variance in gene expression of the lichen-forming fungus L*obaria pulmonaria* onto regulatory (temperature), genetic (10 principal components based on 11 microsatellite loci), acclimatory (site of origin) and biotic (*Plectocarpon lichenum* infection) components based on partial redundancy analysis. Covariance refers to variance shared among variable sets. Shown is the percentage of explained variance.