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CHARACTERISATION OF *CLADONIA RANGIFERINA* TRANSCRIPTOME AND GENOME, AND THE EFFECTS OF DEHYDRATION AND REHYDRATION ON ITS GENE EXPRESSION

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by the Roman numerals I-IV:

I Junttila, S., Lim K-J., and Rudd, S. (2009) Optimization and comparison of different methods for RNA isolation for cDNA library construction from the reindeer lichen *Cladonia rangiferina*. *BMC Research Notes* 2:204

II Junttila, S. and Rudd, S. (2012) Characterization of a transcriptome from a non-model organism, *Cladonia rangiferina*, the grey reindeer lichen, using high-throughput next generation sequencing and EST sequence data. *BMC Genomics* 13:575

III Junttila, S., Laiho, A., Gyenesei, A., and Rudd, S. (2013) Whole transcriptome characterization of the effects of dehydration and rehydration on *Cladonia rangiferina*, the grey reindeer lichen. *BMC Genomics* 14:933

IV Junttila, S., Mason, J., Hercus, R. and Rudd, S. (2015) Preliminary *de novo* assembly and characterization of the lichenized *Cladonia rangiferina* genome. *Manuscript*

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pair
BP	biological process, a GO category
CC	cellular component, a GO category
cDNA	complementary DNA
cDNA-AFLP	cDNA amplified fragment length polymorphism
ChIP	chromatin immunoprecipitation
CNV	copy number variation
CTAB	cetyl-trimethyl-ammonium-bromide
DEG	differentially expressed gene
DNA	deoxyribonucleic acid
EC	enzyme code
EST	expressed sequence tag
F_0	background chlorophyll fluorescence
G6PD	glucose 6-phosphate dehydrogenase
GO	gene ontology
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
ITS	internal transcribed spacer
KEGG	Kyoto encyclopaedia of genes and genomes
MF	molecular function, a GO category
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
NGS	next-generation sequencing
NO	nitric oxide
NRPS	non-ribosomal peptide synthetase
nt	nucleotide
PCR	polymerase chain reaction
PGM	personal genome machine
PKS	polyketide synthase
PSI	photosystem I
PSII	photosystem II
RIN	RNA integrity number
RT-PCR	reverse transcription PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RWC	relative water content
SBS	sequencing by synthesis
SMRT	single molecule real time sequencing system
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
WGS	whole genome resequencing

ABSTRACT

Lichens are symbiotic organisms, which consist of the fungal partner and the photosynthetic partner, which can be either an alga or a cyanobacterium. In some lichen species the symbiosis is tripartite, where the relationship includes both an alga and a cyanobacterium alongside the primary symbiont, fungus. The lichen symbiosis is an evolutionarily old adaptation to life on land and many extant fungal species have evolved from lichenised ancestors. Lichens inhabit a wide range of habitats and are capable of living in harsh environments and on nutrient poor substrates, such as bare rocks, often enduring frequent cycles of drying and wetting. Most lichen species are desiccation tolerant, and they can survive long periods of dehydration, but can rapidly resume photosynthesis upon rehydration. The molecular mechanisms behind lichen desiccation tolerance are still largely uncharacterised and little information is available for any lichen species at the genomic or transcriptomic level. The emergence of the high-throughput next generation sequencing (NGS) technologies and the subsequent decrease in the cost of sequencing new genomes and transcriptomes has enabled non-model organism research on the whole genome level.

In this doctoral work the transcriptome and genome of the grey reindeer lichen, *Cladonia rangiferina*, were sequenced, *de novo* assembled and characterised using NGS and traditional expressed sequence tag (EST) technologies. RNA extraction methods were optimised to improve the yield and quality of RNA extracted from lichen tissue. The effects of rehydration and desiccation on *C. rangiferina* gene expression on whole transcriptome level were studied and the most differentially expressed genes were identified.

The secondary metabolites present in *C. rangiferina* decreased the quality – integrity, optical characteristics and utility for sensitive molecular biological applications – of the extracted RNA requiring an optimised RNA extraction method for isolating sufficient quantities of high-quality RNA from lichen tissue in a time- and cost-efficient manner. The *de novo* assembly of the transcriptome of *C. rangiferina* was used to produce a set of contiguous unigene sequences that were used to investigate the biological functions and pathways active in a hydrated lichen thallus. The *de novo* assembly of the genome yielded an assembly containing mostly genes derived from the fungal partner. The assembly was of sufficient quality, in size similar to other lichen-forming fungal genomes and included most of the core eukaryotic genes. Differences in gene expression were detected in all studied stages of desiccation and rehydration, but the largest changes occurred during the early stages of rehydration. The most differentially expressed genes did not have any annotations, making them potentially lichen-specific genes, but several genes known to participate in environmental stress tolerance in other organisms were also identified as differentially expressed.

TIIVISTELMÄ

Jäkälä on symbioosi, johon kuuluu sieniosakas ja yhteyttävä osakas, joka voi olla joko levä tai syanobakteeri. Joissakin jäkälälajeissa symbioosiin kuuluu kolme osakasta, eli sekä levä että syanobakteeri ovat mukana symbioosissa primääriosakkaan eli sienen kanssa. Jäkäläsymbioosi on evolutiivisesti vanha sopeutuma elämään maan päällä ja monet nykyiset sienilajit ovat kehittyneet jäkälistä. Jäkälät elävät hyvin vaihtelevissa elinympäristöissä ja pystyvät selviämään ankarissakin olosuhteissa sekä vähäravinteisilla kasvualustoilla, kuten paljaan kiven päällä. Elinolosuhteidensa vuoksi monet jäkälälajit joutuvat sopeutumaan säännölliseen kuivumisen ja kastumisen vaihteluun. Enemmistö jäkälistä kestää hyvin kuivuutta ja selviää elossa pitkiäkin aikajaksoja ilman vettä. Kastuessaan jäkälät pystyvät kuitenkin nopeasti jatkamaan yhteyttämistä. Molekulaariset mekanismit, jotka vaikuttavat jäkälien kuivuuden sietoon, ovat edelleen suurelta osin tuntemattomia ja jäkälistä on ylipäänsä vähän tietoa saatavilla genomi- ja transkriptomitasolla. NGS-sekvensointimenetelmien kehittäminen ja sitä seurannut genomi- ja transkriptomisekvensoinnin hinnan lasku ovat mahdollistaneet ei-malliorganismien tutkimisen koko genomin tasolla.

Tässä väitöskirjatutkimuksessa harmaaporonjäkälän, *Cladonia rangiferinan*, transkriptomi ja genomi sekvensointiin, *de novo* assembloitiin ja karakterisoitiin käyttäen NGS-tekniikkaa sekä perinteistä EST-sekvensointitekniikkaa. RNA:n eristysmenetelmiä optimoitiin jäkälästä eristetyn RNA:n saannon ja laadun parantamiseksi. Kastumisen ja kuivumisen vaikutuksia *C. rangiferinan* geenien ilmentymiseen tutkittiin koko transkriptomin tasolla ja geenit, joiden ilmentymistaso muuttui eniten, pystyttiin tunnistamaan.

Cladonia rangiferinan sisältämät sekundäärimetaboliitit heikensivät eristetyn RNA:n laatua ja täten tarvittiin jäkälälle optimoitu RNA:n eristysmenetelmä, jotta pystyttiin eristämään tarvittava määrä hyvälaatuista RNA:ta edullisesti ja riittävän nopeasti. *C. rangiferinassa* aktiivisia biologisia toimintoja sekä reaktioteitä pystyttiin tunnistamaan transkriptomin *de novo* assemblyn avulla. Genomin *de novo* assemblyssä saatiin tulokseksi assembly, joka koostui pääasiassa sieniosakkaan geeneistä. Se oli kuitenkin kohtuullisen laadukas, samankokoinen kuin muista jäkälien sieniosakkaista julkaistut genomit sekä sisälsi suurimman osan tärkeimmistä konservoituneista eukaryoottigeeneistä. Jokaisesta tutkitusta harmaaporonjäkälän kastumis- ja kuivumisvaiheesta pystyttiin tunnistamaan eroja geenien ilmentymisessä, mutta suurimmat erot tapahtuivat kastumisen alkuvaiheissa. Geeneille, joiden erot olivat suurimmat, ei löytynyt sekvenssikuvausta, joten ne saattavat olla jäkäläspesifisiä geenejä. Tutkimuksessa havaittiin eroja myös sellaisten geenien ilmentymisessä, joiden tiedetään osallistuvan erilaisten ympäristöperäisten tekijöiden stressinsietokyvyn muodostumiseen muissa organismeissa.

1 INTRODUCTION

Lichens are symbiotic organisms, which consist of a fungal partner called the mycobiont, and either an algal or a cyanobacterial partner called the photobiont. Some lichen species can include both the alga and the cyanobacterium as symbionts and they are often referred to as tripartite lichens. In all lichen symbioses the fungus is the primary organism. Lichens also contain extensive bacterial communities and it has recently been suggested that in addition to the mycobiont and photobiont, the bacterial community should be included in the definition of the lichen symbiosis. The bacteria associated with chlorolichens, i.e. lichens with an algal photobiont, have been found to possess nitrogen-fixing capabilities (Cardinale et al., 2006, Liba et al., 2006, Grube et al., 2009) and therefore most likely contribute to the growth of the symbiosis. The lichen symbiosis is evolutionarily very old (Yuan et al., 2005) and many extant fungal species have evolved from lichenised ancestors (Lutzoni et al., 2001).

Lichens can reproduce either asexually or sexually. In asexual reproduction both symbionts are dispersed together and there is no need for relichenisation (Wornik and Grube, 2009). However, after codispersion the symbionts can separate and the fungal partner can replace the algal partner with one available in the environment allowing for flexibility and optimisation within the symbiosis (Yahr et al., 2006). When the mycobiont reproduces sexually, the ascospores only contain the fungal symbiont, which then has to find a new symbiotic partner upon localisation to the new habitat. Lichen-forming fungi are often capable of forming some form of symbiosis with several different algal species, although the association with a less compatible photobiotic partner can result only in a loose structure, and the contact between the symbionts is not as intimate as with a more compatible photobiont (Schaper and Ott, 2003). Compatible algal cells can also be acquired parasitically from another lichen species with the same photobiont (Gassmann and Ott, 2000). The combination of sexual and asexual reproduction is often a trade-off between increasing genetic diversity and the cost of finding a new compatible symbiotic partner.

Lichens are widely distributed globally and they often inhabit extreme climates, e.g. deserts and arctic tundras. Many of them are slow growing, but can live for hundreds of years. Lichens are poikilohydric meaning that they cannot control the water levels of their thalli, but their hydration status is totally dependent on the prevailing environmental conditions (Palmqvist, 2000). Many lichen species therefore experience frequent desiccation-rehydration cycles and have adapted to tolerate desiccation well, and to resume metabolic activity quickly upon rehydration even after long periods of desiccation (Aubert et al., 2007). Lichens can also tolerate desiccation better than the isolated symbiotic partners (Kranner et al., 2005). Lichen desiccation tolerance has been studied at the biochemical level (Weissman et al., 2005a, Catalá et al., 2010, Kranner, 2002), but the molecular mechanisms underlying lichen desiccation tolerance are still largely unknown.

Lichens are known to contain a diverse array of secondary metabolites, which chemically often belong to depsides or depsidones, complex aromatic compounds derived from polyketides. The secondary metabolites are produced by the mycobiont and many of these compounds are found uniquely in lichens (Miao et al., 2001). Nitrogen is often a limiting resource for lichens and the excess carbon could be used in the synthesis of secondary carbon metabolites (Palmqvist, 2000), but they are also thought to play a role in different protective mechanisms. Several lichen secondary metabolites have been characterised and they have been detected to exert a variety of effects including e.g. antibiotic, antimycobacterial, antiviral, and anti-inflammatory effects (Ingolfsdottir et al., 1998).

The molecular biological study of lichens has been very limited until recently and has been mainly focused on the taxonomic and phylogenetic studies of different lichen species. However, with the emergence of the massively parallel sequencing and next-generation sequencing technologies, lichen research has also been brought into the genomics era (Wang et al., 2014b, Park et al., 2013a, Park et al., 2013b, Park et al., 2014). This high-throughput DNA sequencing technology has enabled genome and transcriptome characterisation from many non-model organisms (Feldmeyer et al., 2011, Franchini et al., 2011, Diguistini et al., 2009) and the amount of data available in public data repositories is growing fast. The generation of relatively large amounts of sequence data from any organism is currently quite feasible, whereas the analysis of non-model organism data is still challenging and time-consuming. Especially obtaining meaningful annotations can be difficult and require considerable resources.

The grey reindeer lichen, *Cladonia rangiferina*, was used as a model organism in this study. As lichens contain large quantities of secondary metabolites, which can interfere with nucleic acid extraction, firstly an extraction protocol suitable for extraction of sufficient RNA amounts for cDNA library construction was optimised (I). High-throughput next-generation sequencing technology and traditional expressed sequence tag sequencing were used to sequence the transcriptome of *C. rangiferina*, which was consequently *de novo* assembled and characterised (II). Based on the assembled transcriptome sequences a custom microarray was designed and the changes occurring in gene expression during drying and wetting of the lichen thallus were further studied (III). Finally, genomic DNA from the *C. rangiferina* thallus was sequenced, *de novo* assembled and characterised (IV).

2 REVIEW OF THE LITERATURE

2.1 Lichens and the lichen symbiosis

2.1.1 *The nature of the lichen symbiosis*

Lichens consist of two or sometimes three symbiotic partners. However, the taxonomic name of the lichen is the name of the symbiotic fungus, and the photobiont has a separate taxonomic name. Lichen symbiosis is a common occurrence in the fungal kingdom with 20% of all extant fungal species and 40% of the Ascomycota forming lichen symbioses (DePriest, 2004). The Ascomycota include an overwhelming majority of the lichen-forming fungi, as approximately 99% of lichen mycobionts belongs to this group (Oksanen et al., 2013). For algae the lichen symbiosis is not as common as for fungi with an estimated 100 species from 40 genera participating in the symbiosis (DePriest, 2004). No algal genus is as dominant within the photobionts as the Ascomycota is within the mycobionts. The two most common photobiont genera, *Trebouxia* and *Asterochloris*, are present in approximately 35% of all lichen species (DePriest, 2004). Approximately 85% of lichen species are comprised of mycobionts in association with green algal species. An estimated 10% of the lichen-forming fungi form associations with cyanobacteria, and about 3-4% of the symbioses contain both green algal and cyanobacterial symbionts (Honegger, 2008a). Cyanobacteria, which are able to provide both fixed nitrogen and photosynthetically fixed carbon, occur in two distinct types of lichen symbioses. In two-part symbioses, also referred to as bipartite, a continuous layer of photosynthetic and nitrogen-fixing cyanobacterial symbionts is found. In three-part, or tripartite symbioses, warty structures containing nitrogen-fixing cyanobionts coexist with a green alga, which forms the photosynthetic layer (DePriest, 2004).

The nature of the lichen symbiosis is still debated with some researchers seeing it as fungal parasitism of the photobiont and some seeing it as a mutualistic relationship between the mycobiont and the photobiont. The observation that lichens are better adapted to their thermal environment than their symbionts (Sun and Friedmann, 2005) supports the mutualistic view of the symbiosis, and the symbiosis offers an adaptive advantage for both the mycobiont and the photobiont as lichens have been found to better tolerate desiccation than the isolated symbiotic partners (Kranner et al., 2005). This undoubtedly widens the possible living environments for lichens. Regardless of being classified either as a mutualism or a controlled parasitism, the association between the mycobiont and the photobiont is stable, self-supporting and self-reproducing (DePriest, 2004).

Lichen symbiosis is an evolutionarily old adaptation to life on land and above ground, and the formation of a lichen symbiosis between a fungus and a photosynthesising organism may even have been one of the first steps in the colonisation of land by eukaryotes (Heckman et al., 2001). Lichen-like fossils, which were dated 600 million years old by direct lead-lead dating, indicate that fungi

developed symbiotic partnerships with photoautotrophs already before the evolution of vascular plants (Yuan et al., 2005). A cladistics analysis of small subunit ribosomal DNA from lichen-forming and non-lichen-forming fungi (Gargas et al., 1995) and a 6-gene, 420-species phylogeny analysis of Ascomycota (Schoch et al., 2009) suggest that lichenisation has evolved independently multiple times in phylogenetically distant fungal groups. An early study hypothesized that current major Ascomycota fungal lineages of exclusively non-lichen-forming species, taxa such as *Aspergillus* and *Penicillium*, are derived from lichen-forming ancestors (Lutzoni et al., 2001), but the results of more recent studies do not support this hypothesis (Schoch et al., 2009, Gueidan et al., 2008).

2.1.2 *Lichen structure*

In the lichen symbiosis, the fungal hyphae form a three-dimensional structure called the thallus, within which the algal cells are located. Internally stratified lichens exhibit a structure with several layers - an outer gelatinous cortex, an upper algal layer housing the photobiont, and an inner gas-filled medulla (Dyer, 2002). Lichens are often divided into three main categories - crustose, foliose and fruticose - based on the structure of their thallus, but several more detailed categorisations exist within the main categories (Büdel and Scheidegger, 2008).

A majority of lichen species, about 75%, form only small and often inconspicuous thalli, and are commonly referred to as microlichens, whereas only an estimated 25% of lichen-forming fungi form foliose or fruticose (leaf- or shrub-like) symbiotic phenotypes, which are often referred to as macrolichens (Honegger, 1998). Thallus morphologies vary greatly from one lichen species to the next, but the thalli are optimised for water uptake within a specific habitat (Büdel and Scheidegger, 2008). The thallus provides the alga an ideal space for photosynthesis, as the thallus is illuminated and the water level within the algal layer is controlled through proteins called hydrophobins. Hydrophobins function to keep interhyphal spaces water-free and seal the apoplast to enable water translocation (Trembley et al., 2002a). The hydrophobin molecules may also be involved in important aspects of maintaining, functioning and likely establishing of the symbiotic phenotype (Trembley et al., 2002b).

2.1.3 *Lichen bacterial communities*

In recent years, the bacterial communities present in lichen thalli have been studied extensively revealing the complexities of these communities. Although the understanding of the phylogenetic structure and variability of the lichen-associated microbial communities is still limited, bacteria have been suggested to be included in the lichen symbiosis, and they most likely serve distinct functional roles within the symbiosis (Bates et al., 2011). Suggested roles for bacteria include release of nitrogenated compounds such as amino acids, involvement in defence against lichen pathogens and feeders, degradation of parts of lichen thalli to facilitate biomass

mobilisation, and influencing the growth by producing hormones (Grube and Berg, 2009).

Many lichen bacterial communities are dominated by Alphaproteobacteria, with other taxa commonly detected from lichen thalli including Actinobacteria, Firmicutes and Betaproteobacteria (Cardinale et al., 2012, Mushegian et al., 2011, Grube et al., 2009, Cardinale et al., 2008, Hodkinson et al., 2012, Bates et al., 2011, Cardinale et al., 2006). In addition to *Bacteria*, species from the *Archaea* have been found to be associated with rock-inhabiting lichens (Bjelland et al., 2011). Bacterial communities mainly occur in small colonies ranging from a few to a few hundred cells, but can also form a biofilm-like, continuous layer, which can reach an abundance of up to 10^8 cells per thallus fresh weight (Grube et al., 2009, Cardinale et al., 2008).

The bacterial communities are different between chlorolichens and cyanolichens (Hodkinson et al., 2012), most likely due to the nitrogen-fixing capabilities of cyanobacterial symbionts. Bacteria capable of fixing nitrogen have been identified within the lichen bacterial communities, especially in chlorolichens (Cardinale et al., 2006, Grube et al., 2009, Liba et al., 2006). Lichen-associated bacterial communities differ clearly from those in nearby soil (Bates et al., 2011), and the composition of the bacterial communities has been shown to be affected by the lichen species, the pattern of secondary metabolites, and diverse environmental parameters such as geography, the substrate, and the microhabitat conditions (Printzen et al., 2012, Cardinale et al., 2006, Mushegian et al., 2011, Hodkinson et al., 2012).

2.1.4 *Lichen living environment*

Lichens are the dominant species in approximately 8% of terrestrial ecosystems (Larson, 1987) and they can be found in habitats that range from the tropics to the polar regions. In most of these lichen-dominated locations vascular plants are at their physiological limits and the ability of lichens to survive cold, heat and drought stress practically unharmed gains them a distinct ecological advantage (Honegger, 1991). Lichens are able to acclimate their photosynthetic strategy according to the environmental changes present in their habitat thus allowing them to maintain photosynthesis under diverse conditions (MacKenzie et al., 2001). They are known to inhabit some of the harshest environments like the Antarctica and have even been found to survive the extreme conditions of space (de Vera et al., 2004). Lichens are also pioneers inhabiting bare rock or soil, can act as agents of mineral weathering and fine-earth stabilisation, and produce significant amounts of biomass in extreme environments (Beckett et al., 2013).

The community adaptation hypothesis predicts that lichens can adapt to a wide range of thermal regimes by regulating the ratio of primary producers (algae) and consumers (fungi) (Sun and Friedmann, 2005). This ability to balance energy production and usage could explain how lichens are able to colonise such different living environments. Studies on different lichen species and samples from different locations around the world indicate that environmental factors play a role in the

photobiont selection of the mycobiont (Domaschke et al., 2013, Peksa and Skaloud, 2011, Muggia et al., 2013, Yahr et al., 2006). These studies suggest a dynamic relationship between the symbionts within the lichen symbiosis allowing for acclimation of the species in a variety of habitats.

Due to their slow growth and long life spans lichens have been shown to accumulate some compounds present in their environment. While lichens can tolerate desiccation, some species are quite sensitive to air pollution and thus lichens may be used as bioindicators for monitoring air pollution in urban or rural areas (Wolterbeek, 2002, Malaspina et al., 2014, Loppi et al., 2015, Canha et al., 2014). Since lichens effectively accumulate and retain most of the deposited radionuclides from air, they have been used as an indicator of past radioactivity discharges (Lindahl et al., 2004). *Cladonia* species have been used as deposition indicators for transuranium elements after the Chernobyl disaster in Finland (Paatero et al., 1998) and *Cladonia stellaris* has been used to monitor neptunium and plutonium levels in Sweden (Lindahl et al., 2004). *Parmelia sulcata* and *Xanthoria parietina* have been used as bioindicators of ^{137}cesium fallout in the Netherlands (Sloof and Wolterbeek, 1992). Epiphytic lichens have also been shown to be effective biomonitors as accumulator organisms in pollution studies (van Dobben et al., 2001), while *Alectoria sarmentosa*, an epiphytic fructicose lichen, has been used to measure isotopic compositions and total sulphur levels in Canada (Wadleigh, 2003). Proteins associated with cadmium stress in lichens have been identified and they might be used as molecular biomarkers in environmental studies (Rustichelli et al., 2008).

2.1.5 *Lichen reproduction*

Lichen reproduction is being complicated by the separate organisms that participate in the symbiosis. Lichens can either disperse sexually through fungal spore formation in the fruit bodies, or ascomata, or asexually through vegetative propagules and fragmentation. Co-dispersal of symbiotic partners by joint propagules is considered to be an efficient strategy to maintain successful associations in environments where the symbiont is scarce (Wornik and Grube, 2009). Ascospores may also be dispersed from ascomata without an algal partner during sexual reproduction, this promotes new symbiont combinations at each dispersal event (Piercey-Normore and Deduke, 2011).

Sexually reproducing lichen-forming fungi can self-fertilise and this breeding system, also called homothallism, allows these symbiotic organisms to reproduce successfully in harsh environments (Murtagh et al., 2000). Murtagh et al. (2000) suggested ecological functions for homothallism in lichens including genetic stability, high spore output and the ability to develop a lichen population from an individual spore. The genetic stability resulting from self-fertility can be advantageous especially in extreme environments, but lichen species have needed to find a balance between the increase of variation provided by outbreeding and the advantages of self-fertility resulting in the presence of heterothallic or homothallic breeding system in a particular lichen-forming fungi (Seymour et al., 2005). In the *Cladonia* genus it has been found that spores from the same apothecium are not genetically uniform, indicating

heterothallism (Seymour et al., 2005). Reproduction strategies have been studied in *Lobaria pulmonaria*, and the results are somewhat conflicting, with some studies indicating *L. pulmonaria* as predominantly outcrossing species (Walser et al., 2004, Singh et al., 2012) and others as mostly vegetatively reproducing species (Dal Grande et al., 2012, Werth and Scheidegger, 2012). One explanation could be that these studies used samples of different size as *L. pulmonaria* has been found to need a minimum size to form reproductive structures and uses a mixed strategy of early asexual reproduction and later sexual (Martínez et al., 2012).

When the lichen-forming fungus reproduces sexually and disperses ascospores without the algal partner, it needs to find compatible photobionts for relichenisation. Gassmann and Ott (2000) studied the methods of relichenisation in the mycobiont *Ochrolechia frigida* and its photobiont partner *Trebouxia* sp. They found that degenerating thalli can release compatible photobionts in lichen-rich regions and that algal cells from old thalli are likely to be incorporated during lichenisation as evidenced by the presence of different species of *Trebouxia* in one lichen thallus and the presence of the same *Trebouxia* species in different lichen species. Also a parasitic procurement of compatible algal cells from other lichen species is plausible (Gassmann and Ott, 2000, Honegger, 2008b).

2.1.6 *Mycobiont selectivity and relichenisation*

The selectivity of the fungal partner in its choice of an algal partner varies significantly between different lichen-forming fungal species. Some fungi are very species-specific in their choice of photobiont, e.g. *Lecanora conizaeoides* has high specificity for *Trebouxia simplex* (Hauck et al., 2007), whereas other fungal species can lichenise with several different algal species, e.g. *Evernia mesomorpha* can associate with multiple genotypes of *Trebouxia jamesii* (Piercey-Normore, 2006). Several cyanolichen species from Finland were found to be highly selective of their symbiotic partner (Stenroos et al., 2006, Myllys et al., 2007), while only *Lobaria pulmonaria* exhibited flexibility with its association with different *Nostoc* cyanobionts (Myllys et al., 2007). The fungal selection for the algal partner may vary even within the same genus, as observed in *Cladonia gracilis*, *C. multiformis* and *C. rangiferina* (Piercey-Normore, 2004), and the selectivity of numerous lichen-forming fungal species towards the photobiont can be seen as a continuum of intensity on species level (Schaper and Ott, 2003). *Cladonia subtenuis* has been found to associate with four *Asterochloris* algal clades at different sites indicating a dynamic and environment-dependent photobiont selection (Yahr et al., 2006). Peksa and Skaloud (2011) found that environmental conditions affected the selectivity of the mycobiont. In their study particular *Asterochloris* lineages were associated with taxonomically different but ecologically similar lichens, and particularly the exposure of the lichen to sun and rain was significant in differentiating the *Asterochloris* lineages (Peksa and Skaloud, 2011). Ecological diversification and speciation of lichen symbionts in different habitats promoted by e.g. varying physiological backgrounds has been suggested to include a transient phase, where more than one photobiont is present in individual thalli, as observed in *Ramalina farinacea* with the same two algal

Trebouxia species coexisting in every thallus throughout the northern hemisphere (Del Campo et al., 2012). Also in *Xanthoria parietina* and *Anaptychia ciliaris* most thalli harboured several photobiont strains indicating environmental uptake of algae either at the beginning of the symbiosis or later when thallus has already been established and suggesting that promiscuous mycobiont-photobiont associations in lichen symbioses with *Trebouxia spp.* are more frequent than currently recognised (Dal Grande et al., 2014). Muggia et al. (2013) also detected several photobiont genotypes within individual *Protoparmeliopsis muralis* and additionally algal communities colonising the thalli. These algal communities contain taxa, which are known to form symbiotic associations with other fungi, suggesting that lichen surfaces can act as a potential temporary niche for free-living algae, possibly facilitating the establishment of new lichen symbioses within the neighbouring area (Muggia et al., 2013).

The formation of the lichen symbiosis has been studied using cultured mycobionts and photobionts, and stages within the re-lichenisation process have been identified. These stages of relichenisation were described by Galun in 1988 and include the initial contact followed by the formation of mucilage and the development of soredia-like structures embedded in a gelatinous matrix (Galun, 1988). The formation of soredia-like structures with a compatible photobiont has been observed to lead into the establishment of larger prethallus units (Trembley et al., 2002c). A distinctive phenotypic response - the enveloping of the algal cells by the fungal cells and a contact between the two cell types - to a compatible algal species has been observed for different lichen-forming fungal species that does not develop when the fungus is in contact with a non-compatible or less compatible organism or growing axenically (Trembley et al., 2002c, Joneson and Lutzoni, 2009, Schaper and Ott, 2003). However, a fungus can establish a temporary symbiotic association with a less compatible algal partner, if a more compatible algal symbiont is not available. This has been demonstrated by Schaper and Ott (2003), who studied the selectivity of the fungus *Fulgensia bracteata* towards a variety of potential photobionts. They observed that when highly compatible symbionts come into contact the first step of recognition, which is the formation of mucilage, can occur and is then followed by primary lichenisation. Whereas when less compatible symbionts come into contact the formation of mucilage may be present, but the subsequent development of the association may involve only a loose structure between the fungus and the alga and the contact between the symbionts is not as intimate as with a more compatible photobiont (Schaper and Ott, 2003).

The proteins involved in the symbiont recognition and establishment of the symbiosis have been studied. In one of the earliest studies, Bubrick and Galun (Bubrick and Galun, 1980) isolated a protein fraction from the lichen *Xanthoria parietina* that was capable of discriminating between strains of cultured photobionts and was hypothesised to be involved in the initial selection of potentially suitable photobionts. The *Xanthoria*-protein was found to be present in both the intact thallus and the cultured mycobiont making its biosynthesis independent of the lichen symbiosis (Bubrick et al., 1981). Later, proteins called lectins have been identified in several lichen-forming fungal species and they are known to play a role in the

recognition of compatible algal cells in both chlorolichens and cyanolichens (Sacristán et al., 2007). The retention of the fungal lectin on the algal cell surface is ligand-dependent, and lichen formation between the algal and fungal cells will occur only when the algal ligand is specific for the fungal lectin (Legaz et al., 2004). A specific stereochemical fit between the components on the photobiont surface and their counterparts on the mycobiont cell wall have been suggested to provide the required selectivity for recognition and initial interaction between compatible symbionts (Elifio et al., 2000). Wang et al. (2014b) identified three lectins in the lichen-forming fungus *Endocarpon pusillum*, which increased their expression levels significantly during co-culturing with the photobiont *Diplosphaera chodatii* and were essential for symbiont recognition. As all of these essential lectins lacked a signal peptide in their amino acid sequences, they suggested that lectins without a signal peptide may play a major role in symbiotic recognition (Wang et al., 2014b).

Although typical phenotypic responses to relichenisation have been identified, very little is known of the genes involved in the formation of lichen symbiosis. Trembley et al. (2002) used cDNA-AFLP and autoradiography to observe that many mycobiont and photobiont genes were down regulated in the early stage of relichenisation between *Baeomyces rufus* and *Elliptochloris bilobata* and only a few genes were induced. They therefore concluded that the down regulation of gene expression seemed to be significant for the establishment of lichenisation (Trembley et al., 2002c). The expression levels were however only based on the visual inspection of the autoradiographs, and no quantitative expression levels were presented. Contrary to the Trembley et al. (2002) results, Joneson et al. (2011) found a number of genes that were up regulated during the early stages of lichenisation between *Cladonia grayi* and *Asterochloris* sp. Induced fungal genes showed sequence similarity to genes involved in self and non-self recognition, lipid metabolism, and negative regulation of glucose repressible genes, as well as to a putative D-arabitol reductase and two dioxygenases, whereas the up regulated algal genes included a chitinase-like protein, an amino acid metabolism protein, a dynein-related protein and a protein arginine methyltransferase. These results also provided the first evidence that extracellular communication without cellular contact can occur between lichen symbionts (Joneson et al., 2011). Expression of lec-1, a gene encoding galectin-like protein, was up regulated by two orders of magnitude in portions of the lichen thallus that included both symbionts when compared to tissues lacking photobionts (Miao et al., 2012). This demonstrated that lec-1 expression is influenced by the presence of the photobiont. These contrasting results show that the information available on gene expression during lichenisation is not yet sufficient to draw definite conclusions and especially data at the whole genome or transcriptome level is still very scarce. The specific relationship of *Endocarpon pusillum* with its photobiont *Diplosphaera chodatii* was reflected in the expansion and contraction of certain multigene families within one of the few published lichen-forming fungal genome assemblies (Wang et al., 2014b).

2.1.7 *Lichen secondary metabolites*

Lichens are known to produce a plethora of secondary metabolites many of which are unique to them. Several of these secondary metabolites belong to the polyketide family of molecules and some of them, such as depsides and depsidones, are rarely found elsewhere (Miao et al., 2001). These metabolites can amount to between 0.1% and 10% of the dry weight of the thallus, and sometimes even up to 30% (Palmqvist, 2000) with the season strongly influencing the amount of most carbon based secondary compounds (Gauslaa et al., 2013). Lichens with nitrogen-fixing cyanobacterial photobionts usually lack secondary carbon metabolites, and it has therefore been hypothesised that the synthesis of complex secondary carbon compounds might simply be a way of making use of excess carbon when nitrogen is a limiting source (Palmqvist, 2000). Lichen secondary compounds can have some biological function, for instance, *Lobaria pulmonaria* deposits melanic compounds in the outer layer of the upper cortex and these compounds act as a sun screen for the photobiont (Gauslaa and Solhaug, 2001). It has also been suggested that depsides and depsidones have water relations-based functions (Armaleo et al., 2008). In addition secondary carbon products have been found to serve a defensive role against herbivores (Pöykkö et al., 2005, Nybakken et al., 2010).

The secondary metabolites are predominantly produced by the mycobiont. Although metabolites can be synthesised when the symbiotic fungus is cultured axenically (Culberson and Armaleo, 1992), the compounds produced by the cultured mycobiont may differ when produced in culture or within the thallus (Huneck, 1999, Fox and Huneck, 1969, Hamada and Ueno, 1987). For example, the cultured mycobiont of *Physconia distorta* produced fatty acids as the main secondary compound, whereas the symbiotic thallus contains mostly phenolic compounds (Molina et al., 2003), and the production of secondary metabolites by the cultured mycobiont of *Heterodea muelleri* required stressful conditions such as shortwave ultraviolet light and low temperature (Hager and Stocker-Worgotter, 2005). The cultured mycobiont of *Xanthoria elegans* has been shown to produce typical lichen substances and pigments, which were also partially released into the culture medium, whereas axenically cultured mycobiont *Lecanora rupicola* produced its typical lichen substances only after resynthesis with its algal partner (Brunauer and Stocker-Worgotter, 2005). pH level and media composition have been found to influence the production of secondary products in the cultured lichen-forming fungus *Ramalina dilacerata* (Timsina et al., 2013). The complete chemosyndrome of the lichen-forming fungus *Lobaria spathulata* has been achieved in culture (Stocker-Worgotter and Elix, 2002).

Although the mycobionts of cyanolichens rarely synthesise secondary metabolites, the cyanobacteria in these symbioses are known to produce secondary metabolites. Microcystins are toxic compounds associated almost exclusively with planktonic cyanobacteria. While these potent toxins are commonly associated with algal blooms in aquatic ecosystems, they have also been discovered from a lichen-associated terrestrial cyanobacterium (Oksanen et al., 2004). Cyanobacterial symbionts of

terrestrial lichens capable of producing microcystins have been detected from specimens all over the world and from different lichen lineages (Kaasalainen et al., 2012).

Lichen secondary metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects (Gulluce et al., 2006, Bezivin et al., 2003, Oettl et al., 2013, Cheng et al., 2013, Ingolfsdottir et al., 1998, Muller, 2001). The diverse effects of secondary metabolite extracts from several different lichen species have been studied on a variety of targets and substances. For example methanol extracts of *Hypogymnia physodes* have been shown to have anti-growth effects at relatively lower concentrations while relatively higher concentrations are required for genotoxic and cytotoxic activity on two different human breast cancer cell lines (Ari et al., 2012). Extracts from *Usnea ghattensis* (Behera et al., 2005) and *Parmotrema reticulatum* (Ghate et al., 2013) have been shown to exert antioxidative activity. The methanolic extracts of *Graphis guimarana, G. nakanishiana* and *G. schizograpta* natural thalli and *in vitro* cultures were found to inhibit tyrosinase, xanthine oxidase and to scavenge superoxide (Behera et al., 2006). Acetone extracts of three lichen species (*Parmelia sulcata, Cladonia rangiferina* and *Lobaria pulmonaria*) have the ability to degrade prion protein from transmissible spongiform encephalopathy infected hamsters, mice and deer (Johnson et al., 2011). Anziaic acid, a depside from the lichen *Hypotrachyna* sp., can act as a topoisomerase inhibitor, which could be used for antibacterial and anticancer therapy (Cheng et al., 2013).

Usnic acid is a secondary metabolite uniquely found in lichens that is especially abundant in genera such as *Alectoria, Cladonia, Evernia, Lecanora, Ramalina* and *Usnea*, and it is the most studied lichen secondary metabolite (Araújo et al., 2015). Many lichens and extracts containing usnic acid have been utilised for medicinal, perfumery, cosmetic as well as ecological applications. Usnic acid has been shown to exhibit antibacterial (Ingolfsdottir et al., 1998), antiviral (Perry et al., 1999), antiproliferative (Kristmundsdottir et al., 2002), and anti-inflammatory activity (Vijayakumar et al., 2000) as well as to demonstrate ecological effects such as antigrowth, antiherbivore and anti-insect properties (Ingolfsdottir, 2002). It has also been found to work as a UVB filter and protect against UVB irradiation (Rancan et al., 2002). Usnic acid possesses significant antistaphylococcal activity in both *in vitro* as well as *in vivo* conditions and it exerts its effect through the disruption of the bacterial cell membrane (Gupta et al., 2012). Usnic acid has been observed to inhibit the growth and proliferation of human cancer cells through disruption of mitochondrial membrane potential (Einarsdottir et al., 2010). The loss of mitochondrial membrane potential is the result of usnic acid's ability to shuttle protons across membranes against the gradient (Bessadottir et al., 2012). Additionally, usnic acid has been shown to act as a generic repressor of RNA transcription (Campanella et al., 2002). Interference with RNA synthesis may be a general mechanism for its antimicrobial activity, with additional direct mechanisms, like impairment of DNA replication in *Bacillus subtilis* and *Staphylococcus aureus* (Maciąg-Dorszyńska et al., 2014). Usnic acid has been evaluated against parasites of *Plasmodium berghei*, the malaria parasite, and it has

shown therapeutic and prophylactic potential as antibacterial and antiplasmodial agents (Lauinger et al., 2013).

2.1.8 *Lichen desiccation tolerance*

Lichens are poikilohydric organisms and are unable to control their water level, hence lichen growth is strongly dependent on environmental water availability (Palmqvist, 2000). A large proportion of lichen species live in environments where they are adapted to frequent cycles of drying and wetting and can face long periods of extreme desiccation. A majority of lichen species are desiccation tolerant, but certain lichen species, e.g. *Pseudocyphellaria dissimilis* from New Zealand, growing in very humid habitats are relatively desiccation-sensitive (Kranner et al., 2008). Epilithic and terrestrial lichens are hydrated only 35-65% of time during the year, depending on the lichen species and habitat (Heber et al., 2006). Lichens have therefore evolved to survive in habitats that frequently exhibit adverse living conditions and they employ different protective and activating mechanisms during the rehydration-desiccation cycle as summarised in Figure 1. During prolonged severe desiccation lichens can adopt a state called anhydrobiosis, in which all metabolic processes are reversibly ceased allowing for the organism to survive long periods of complete desiccation (Clegg, 2001). Upon favourable hydration conditions anhydrobiosis is reversed and normal metabolic processes resumed.

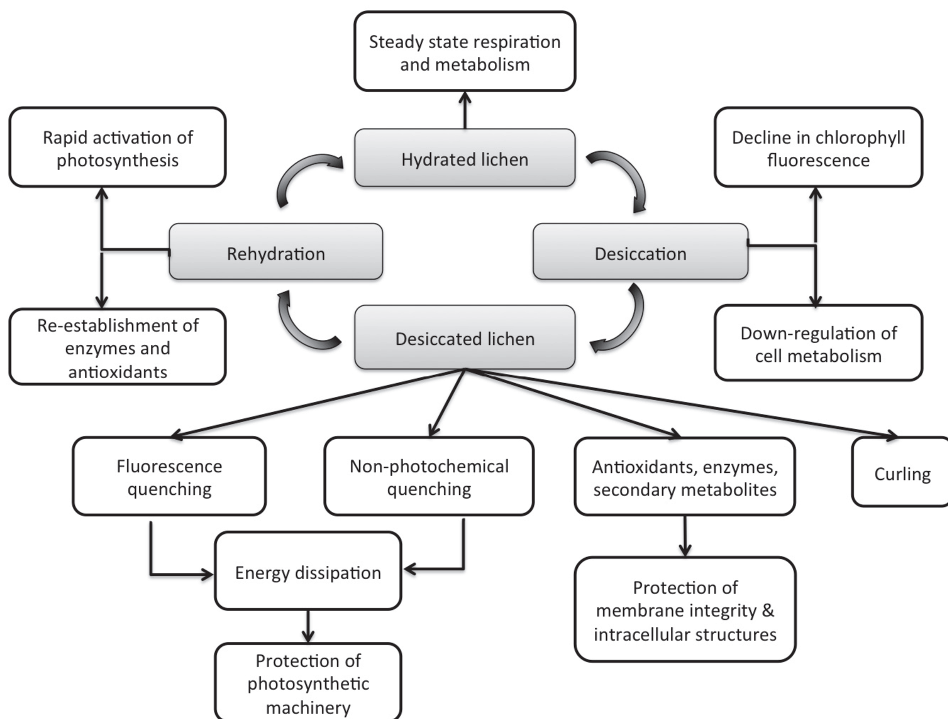

Figure 1. Mechanisms in lichen desiccation-rehydration cycle.

Chlorolichens are able to recover photosynthesis after desiccation through rehydration from water vapour adsorption, whereas cyanolichens always require the addition of liquid water to recover from desiccation (Palmqvist, 2000). Lichens are also specialised in utilising water from different sources such as dew and fog (Hartard et al., 2009). Some desert lichen species can even obtain the required water intake from the air humidity during the night. The length of the dehydration can affect the rehydration efficiency as some lichen species require liquid water in order to completely recover after longer desiccation periods (Kranner, 2002). Chlorolichens have a photosynthetic advantage when thallus water contents are low or the thalli are in equilibrium with atmospheric humidity, whereas cyanolichens are at an advantage when thallus water contents are very high (Green et al., 1993). Both chlorolichens and cyanolichens have shown higher relative growth rates under laboratory conditions when they were hydrated day and night compared to hydration in light only suggesting that nocturnal metabolic activity is important for lichen growth (Bidussi et al., 2013).

How desiccation-tolerant a lichen is, affects how quickly the species is able to resume metabolic activities after desiccation. Desiccation-tolerant organisms can endure very low water contents, as they can survive even at water content of 10% (w/w) (Fernández-Marín et al., 2010). Production of high levels of extracellular superoxide radical (O_2^-) and the existence of an inducible oxidative burst in response to desiccation stress are best developed in lichen species growing in wet microhabitats, whereas the extracellular production of O_2^- is almost absent from desiccation-tolerant lichens (Beckett et al., 2003, Minibayeva and Beckett, 2001). Desiccation-tolerant lichen species can rapidly recover from desiccation (Kranner, 2002, Kranner et al., 2003, Kranner et al., 2005, Weissman et al., 2005b); respiration can be resumed within seconds and photosynthesis within minutes (Aubert et al., 2007). However, desiccation-tolerance comes at a cost and is almost certainly one of the reasons for the slow growth rates of lichens compared to vascular plants, but on the other hand it enables lichens to live in places where higher plants cannot survive (Kranner et al., 2008).

Desiccation tolerance in lichens has been studied at the biochemical level by assaying the activity of enzymes or the levels of other metabolites. Kranner (2002) measured reduced and oxidised glutathione, glutathione reductase (GR) and glucose-6-phospate dehydrogenase (G6PDH) in *Pseudevernia furfuracea*, *Peltigera polydactyla* and *Lobaria pulmonaria* during short- and long-term desiccation and rehydration. GR and G6PDH were measured also from *Cladonia vulcani* and its axenically grown symbiotic partners during desiccation and rehydration (Kranner et al., 2005). GR and G6PDH along with superoxide dismutase (SOD) and catalase have been studied in *Ramalina lacera* during rehydration (Weissman et al., 2005a), and Aubert et al. (2007) have analysed the metabolite profile of *Xanthoria elegans* thalli during hydration and dehydration using nuclear magnetic resonance. Chromatographic analyses have been used to study plastid pigments, such as xanthophyll cycle pigments, and antioxidants in e.g. *Parmelia quercina* (Calatayud et al., 1997), *P. acetabulum* (Calatayud et al., 1997), *Ramalina farinacea* (Calatayud et al., 1997), *Pseudevernia furfuracea* (Calatayud et al., 1997, Kranner et al., 2003), *Evernia*

prunastri (Calatayud et al., 1997), *Lobaria pulmonaria* (Kranner et al., 2003, Stepigova et al., 2008, Heber et al., 2010, Fernández-Marín et al., 2010) and *Peltigera polydactyla* (Kranner et al., 2003).

In the desiccated state lichens have to convert all absorbed light energy into heat in order to avoid oxidative damage. Therefore efficient photoprotection mechanisms are important, while fluorescence quenching keeps the photosynthetic machinery functional for as long as possible. Energy dissipation in photosystem II (PSII) reaction centres and zeaxanthin-dependent energy dissipation protect chlorolichens from photo-oxidation in the absence of water (Heber et al., 2006). The dissipation of excess energy can be studied by measuring the levels of background chlorophyll fluorescence (F_0), as F_0 quenching signals the activation of energy dissipation (Heber et al., 2010). Hence, fluorescence measurements have been used in several studies to measure chlorophyll fluorescence during desiccation and rehydration in e.g. *Lobaria pulmonaria* (Chakir and Jensen, 1999, Jensen et al., 1999, Stepigova et al., 2008, Heber et al., 2010, Fernández-Marín et al., 2010), *Hypogymnia physodes* (Jensen et al., 1999), *Peltigera aphthosa* (Jensen et al., 1999, Maksimov et al., 2014), *Cetraria islandica* (Hajek et al., 2001), *Ramalina lacera* (Weissman et al., 2005b), *R. farinacea* (Catalá et al., 2010), *R. yasudae* (Miyake et al., 2011), *Lasallia pustulata* (Hajek et al., 2006), *Umbilicaria hirsuta* (Hajek et al., 2006), *Parmelia sulcata* (Veerman et al., 2007, Slavov et al., 2013), *Parmelina tiliacea* (Oukarroum et al., 2012), *Physciella melancha* (Komura et al., 2010), *Parmotrema tinctorum* (Miyake et al., 2011) and *Grapis tenella* (Miyake et al., 2011).

The effect of desiccation to chlorophyll fluorescence in lichens can be divided into two phases; during mild desiccation fluorescence decreases slowly and F_0 levels remain relatively constant, whereas during severe desiccation chlorophyll fluorescence declines more steeply along with a considerable decrease in F_0 (Calatayud et al., 1997, Chakir and Jensen, 1999). At the very initial stages of dehydration of fully hydrated thalli chlorophyll fluorescence has been observed to increase (Hajek et al., 2001). The decrease in F_0 during severe water stress is the result of changes in the organisation of the antenna apparatus of PSII (Jensen et al., 1999). In the desiccated state lichens can also protect the manganese cluster of the photosynthetic machinery against heat-induced destruction (Oukarroum et al., 2012), although the most dramatic target of quenching is PSII, which produces negligible levels of fluorescence in desiccated lichens, and this fluorescence decay is dominated by short lifetime, long-wavelength component energetically coupled to PSII (Veerman et al., 2007). In some lichen species, desiccation causes a thylakoid-membrane rearrangement, which brings into direct contact the PSII and PSI units enabling an efficient energy transfer from PSII to PSI, where the transferred energy is then quenched highly efficiently due to the formation of a long-lived $P700^+$ state, and as a consequence, both PSII and PSI are protected very efficiently against photodestruction (Slavov et al., 2013).

A highly effective mechanism of photoprotection employing desiccation-induced loss of chlorophyll fluorescence and of light-dependent charge separation has been found in lichens (Heber, 2008). The activation of this mechanism is proposed to be

based on desiccation-induced conformational changes of a pigment-protein complex, which results in flexibility of photoprotection (Heber et al., 2010). Desiccation-induced conformational changes of a chlorophyll protein complex result in the fast radiationless dissipation of absorbed light energy and according to Heber et al. (2007) this mechanism of photoprotection is more effective in preventing photo-oxidative damage than other mechanisms of energy dissipation, which require light for activation such as zeaxanthin-dependent energy dissipation or quencher formation within the reaction centre of PSII (Heber et al., 2007). Komura et al. (2010) suggested that lichens possess a new type of quenching mechanism, such as the chlorophyll aggregation in light-harvesting complex II or a new type of quenching in PSII core antenna (Komura et al., 2010). Evidence for the existence of two distinct dissipation components of excess light energy in desiccated lichen has been found and the de-excitation by the two quenching channels might be a lichen-specific protection mechanism that has evolved for survival in highly desiccated environments (Miyake et al., 2011). Eventually, the PSII reaction is totally inhibited when photosynthesis is completely inhibited by desiccation (Kosugi et al., 2009).

Lichens are known to additionally prevent damage from sunlight by physically curling their thalli. Natural curling is one strategy to reduce the chance for serious photoinhibition in desiccated *Lobaria pulmonaria* thalli during high light exposures (Bartak et al., 2006). In *Parmelia sulcata* about 20% of the fluorescence quenching was attributed to the sunshine effect induced by structural changes in the lichen thallus (Veerman et al., 2007).

Lichens use also non-photochemical quenching to protect PSII from damage during desiccation, and the level of protection can be measured as an increase in the non-photochemical quenching. Mechanisms involved in the non-photochemical energy dissipation pathways include zeaxanthin formation, thermal dissipation and production of antioxidants (Hajek et al., 2006). The xanthophyll cycle is the conversion of violaxanthin via antheroxanthin to zeaxanthin, and according to results from Fernandez-Marin et al. (2010), the de-epoxidation of violaxanthin to zeaxanthin occurs only after slow dehydration and that zeaxanthin, which is synthesised in the dark, prevents photoinhibition when rehydrated tissues are illuminated. This study also demonstrated the full and reversible operation of the xanthophyll cycle by desiccation-rehydration regime in the dark (Fernández-Marín et al., 2010). However, zeaxanthin was not formed when desiccation was established rapidly, possibly explaining the contradiction with the results obtained by Heber et al. (2010), who concluded that zeaxanthin formation did not contribute to the increase of photoprotection by desiccation in the light. An earlier study by Calatayud et al. (1997) had observed an increase in antheraxanthin and zeaxanthin during lichen desiccation indicating that the de-epoxidised xanthophyll cycle pigments are involved in a photoprotective mechanism, which dissipates excess energy during desiccation (Calatayud et al., 1997). Stepigova et al. (2008) found that irradiance during hydration influences lichens' ability to cope with high irradiance in the following desiccation period. The high zeaxanthin level and the de-epoxidation state of xanthophyll cycle pigments

remain stable throughout the high light period in the dehydrated thalli and facilitate the dissipation of excess energy during the rehydration phase (Stepigova et al., 2008).

In addition to the xanthophyll cycle pigments, compounds such as gluconate 6-P and glutathione, and enzymes such as SOD, catalase, GR, and G6PDH have been found to be associated with lichen desiccation tolerance. Gluconate 6-P accumulates during desiccation due to activation of the oxidative pentose pathway, in response to a need for reducing power (NAPDH) during the dehydration-triggered down-regulation of cell metabolism (Aubert et al., 2007). Kranner (2002) detected an almost complete oxidation of the reduced glutathione (GSH) during desiccation, whereas rehydration caused rapid reduction of oxidised glutathione (GSSG). The reduction of GSSG during rehydration additionally correlated with the re-establishment of the predesiccation activity of G6PDH, a key enzyme of the oxidative pentose shunt (Kranner, 2002). Gluconate 6-P is abundant in the desiccated thallus, but is metabolised immediately after hydration (Aubert et al., 2007). Weissman et al. (2005a) found that the activities of SOD, catalase, GR and G6PDH are decreased upon rehydration, and suggested as reasons an inactivation of the enzymes by reactive oxygen species (ROS), an increase in activities during dehydration, or a degradation of total cellular proteins during desiccation and their resynthesis upon rehydration (Weissman et al., 2005a). G6PDH is also thought to provide NADPH during the first stages of rehydration, when photosynthesis is not yet possible (Kranner, 2002).

The ability to rapidly re-establish the enzymatic and antioxidant pools upon rehydration is indicative of a desiccation-tolerant lichen species (Kranner, 2002, Kranner et al., 2003). Additionally, in desiccation-tolerant lichen species ROS scavenging and photoprotective machinery, as well as membrane integrity, are maintained functional during desiccation (Kranner et al., 2005, Weissman et al., 2005b). The desiccation-induced fluorescence loss is rapidly reversible under rehydration, and although reaction centres of PSII lose activity on dehydration, they regain it upon hydration (Heber and Shuvalov, 2005). Pools of polyols and other secondary metabolites are likely to contribute to the protection of cell constituents like nucleotides, proteins, and membrane lipids, and to preserve the integrity of intracellular structures during desiccation (Aubert et al., 2007). Fernandez-Marin et al. (2010) have suggested that zeaxanthin can potentially be involved in the maintenance of the integrity and function of the photosynthetic apparatus during desiccation, which is important for the rapid re-establishment of photosynthesis during rehydration.

Although ROS are formed during desiccation, bursts of intracellular production of ROS and NO are linked with the recovery of photosynthesis after desiccation and the formation of ROS is associated with both symbiotic partners (Weissman et al., 2005b). The production of intracellular ROS is caused by electron leakage and NO participates in chlorophyll photoprotection and stabilisation during rehydration (Catalá et al., 2010). Hydration of dry thallus results in an initially high rate of respiration with peak rates one to three times higher than steady-state rates, which are reached 3-7 hours after hydration (Sundberg et al., 1999).

Antioxidant and photoprotective mechanisms have been studied both in the intact lichen *Cladonia vulcani* and in its isolated symbiotic partners, and the lichen has been found to be more desiccation tolerant than the isolated symbiotic partners (Kranner et al., 2005). Interaction with the mycobiont has been found essential for the desiccation-induced non-photochemical quenching to work, as photobionts have been observed to lose the ability for non-photochemical quenching when grown axenically (Kosugi et al., 2013). The improved desiccation-tolerance provides a clear advantage to the lichenised symbiotic state expanding the range of potential habitats. Controlling the damage caused by ROS and the improvement of protection mechanisms, e.g. the quenching of photoinhibition, were very important for the evolution of lichens (Kranner et al., 2008).

The responses of the most common algal symbiont, *Asterochlrois erici*, to desiccation have also been studied independent of the lichen symbiosis. Gasulla et al. (2013) conducted proteomic and transcript analyses, which suggested that desiccation tolerance in *A. erici* is achieved mainly by constitutive mechanisms. These mechanisms appear to involve protein stability, structural cell recovery, protein targeting and degradation, but *A. erici* can also employ a rehydration-induced change in gene expression. For the free-living alga, the rate of dehydration had a more pronounced effect on the recovery of the photosynthetic activity upon rehydration than the length of the desiccation (Gasulla et al., 2009).

Only little research of lichen desiccation tolerance has been done in the molecular level, and the genes involved in desiccation tolerance are still mostly unknown. At least 93 genes involved in drought resistance mechanisms were identified in the genome of *Endocarpon pusillum* (Wang et al., 2014b). Genes involved in regulating osmotic pressure, correcting protein misfolding and scavenging the reactive oxygen species were identified and could be related to survival in extreme drought environment. This situation will hopefully change in the near future with the application of the new high-throughput sequencing techniques to lichen desiccation tolerance research.

2.1.9 *Cladonia rangiferina*

Cladonia rangiferina (L.) Weber ex F.H. Wigg or grey reindeer lichen is a fruticose lichen belonging to the Cladoniaceae family of the Lecanoromycetes, which is among the largest and most diverse groups of lichen-forming fungi (Stenroos et al., 2002). The mycobiont of the symbiosis is *C. rangiferina* and the photobiont is a green alga *Asterochloris* sp. *C. rangiferina* is abundant in northern Europe and often lives on rocky outcrops alongside *C. stellaris*, *C. arbuscula* and *C. mitis*. *Cladonia rangiferina* has a wide ecological distribution from the Arctic to the tropics and it has been suggested that community adaptation directly contributes to the cosmopolitan distribution of *C. rangiferina* (Sun and Friedmann, 2005).

Two novel abietane diterpenoids, hanagokenols A (1) and B (2) have been isolated from *C. rangiferina* (Yoshikawa et al., 2008), and it also contains fumarprotocetraric

acid and atranorin. Extracts from *C. rangiferina* have also been found to degrade prion protein from transmissible spongiform encephalopathy infected hamsters, mice and deer (Johnson et al., 2011).

2.1.10 *Modern molecular biological methods in lichen research*

Lichens have until recently been rather neglected in the genomics area of research. Most of the molecular workflows and resources optimised for lichen species have been aimed at the needs of the taxonomists who need only small amounts of starting material and work mainly on specific nuclear and mitochondrial DNA marker genes. Thus high-throughput genomics and transcriptomics methods are not yet routinely used for lichen species, although an increasing number of studies exploiting these new methods are being published.

Polyketide biosynthesis pathways in lichens have been earlier studied using traditional molecular genetic techniques such as PCR, genomic library construction and heterologous expression (Miao et al., 2001). The diversity of non-reducing polyketide synthase (PKS) genes in members of the lichenised Pertusariales has been studied (Schmitt et al., 2005), individual PKS genes from *Solorina crocea* (Sinnemann et al., 2000, Gagunashvili et al., 2009), *Xanthoparmelia semiviridis* (Chooi et al., 2008), *Xanthoria elegans* (Brunauer et al., 2009), and *Dirinaria applanata* (Valarmathi et al., 2009) have been cloned and characterised, and a PKS gene cluster responsible for the biosynthesis of grayanic acid has been identified from *Cladonia grayi* (Armaleo et al., 2011).

In addition to PKS genes, also hydrophobin genes have been studied in lichens. Hydrophobin gene XPH1 from *Xanthoria parietina* has been characterised and its gene expression within the thallus was studied using *in situ* hybridisation (Scherrer et al., 2002). Hydrophobin genes DGH1, DGH2 and DGH3 from *Dictyonema glabratum* have been identified (Trembley et al., 2002b) and their expression and localisation within the thallus studied (Trembley et al., 2002a).

Park et al. (2013c) have developed a method to transform *Umbilicaria muehlenbergii,* via the use of *Agrobacterium tumefaciens.* This new method enables transformation-mediated mutagenesis in both targeted and random manners also for lichen mycobionts (Park et al., 2013c), although it remains to be seen whether this technique can be readily applied to a wide range of mycobionts.

Although whole transcriptome level research in lichens is very limited, some studies on the differential expression of a restricted set of genes in different lichen species have been published. The symbiosis between *Cladonia grayi* and the alga *Asterochloris* sp. was used to determine fungal and algal genes up regulated *in vitro* in the first two stages of lichen development using quantitative PCR to verify the expression levels of 41 and 33 candidate fungal and algal genes, respectively (Joneson et al., 2011). Development of the symbiotic association in the bipartite lichen *Pseudocyphellaria crocata* has been investigated by characterising two regions of the

thallus. Differential display and quantitative RT-PCR techniques indicated that gene expression was altered in the lichen centre compared with the margin providing evidence for increased heterocyst formation in the thallus margin compared with the centre (Chua et al., 2012). Additionally, the expression of the *psbA* gene in the photobiont of *Xanthoria parietina* has been studied by semi-quantitative RT-PCR (Sen et al., 2014).

In recent years, some studies have been published where modern, high-throughput methods have been used for sequencing lichen genomes (Park et al., 2013a, Wang et al., 2014b), metagenomes (Hodkinson et al., 2012, Kampa et al., 2013) or studying different lichen molecular mechanisms (McDonald et al., 2013). These studies take advantage of various next-generation sequencing applications and bioinformatics analyses, most common of which are described in the next section.

2.2 Next-generation sequencing technology

Massively parallel sequencing or next-generation sequencing (NGS) is a recently developed technology, which allows the sequencing of millions of nucleotide fragments simultaneously. The first NGS technology was described by Margulies et al. in 2005 (Margulies et al., 2005). NGS is an extremely high-throughput technology capable of generating very large amounts of sequence data at single nucleotide resolution in a relatively short time. NGS has revolutionised genomics, enabled several new applications and trivialised research projects, like genome resequencing, that were considered huge tasks only a decade ago. However, the technology has created new bottlenecks such as efficient data storage and processing.

This technology has been used in lichen research, although the number of published studies using NGS is still very modest. The mitochondrial genomes from the mycobionts of two terricolous lichens, *Peltigera membranacea* and *P. malacea*, have been determined using metagenomic approaches, including RNA sequencing (Xavier et al., 2012). The draft genome sequences for the mycobionts of *Caloplaca flavorubescens, Cladonia metacorallifera* and *C. malicenta* have been published (Park et al., 2013b, Park et al., 2014, Park et al., 2013a). The genome of the mycobiont of *Endocarpon pusillum* has been sequenced and during the study the expression levels of some symbiosis-related genes were measured using quantitative RT-PCR when *E. pusillum* pre-contacted with its photobiont *Diplosphaera chodatii* (Wang et al., 2014b). The lec-1 genes, encoding a galectin-like protein, were identified in the metagenome sequence of *Peltigera membranacea*, and analysis of the gene structure indicated that it was derived from the mycobiont (Miao et al., 2012). NGS has been used to assess the presence or loss of ammonium transporter/ammonia permease genes in the assembled genomes of eight lichen-forming fungi (McDonald et al., 2013), and the metagenome of *Peltigera membrenacea* has been sequenced and *de novo* assembled for the identification of PKS gene clusters (Kampa et al., 2013). NGS has also been used to study the bacterial communities within lichens (Hodkinson et al., 2012).

The main NGS technologies currently are Illumina's sequencing by synthesis (SBS), Ion Torrent's semiconductor sequencing and Pacific Biosciences' single molecule real time sequencing system (SMRT). Roche's pyrosequencing was the first NGS technology in the market, and although used extensively during the emergence of NGS technologies, is now used less extensively. All of the main technologies, with the exception of the SMRT sequencing, rely on amplification of the sample and a construction of a representative sequencing library before sequencing. During the library preparation synthetic oligonucleotide barcodes can be added to the sequenced libraries allowing for the sequencing of several samples in one run.

In Roche's technology the incorporation of a complementary base to the single-stranded amplified library fragment is detected by the emission of light following the release of a pyrophosphate group from the sequenced nucleotide chain. Nucleotides are added to the template sequentially in a specific order, thus the identity of the incorporated nucleotide is known and the sequence of the DNA strand can be deciphered based on the emissions of light (Ansorge, 2009). A relatively high cost compared to the other methods and a generally lower reading accuracy in stretches of consecutive identical bases, called homopolymer stretches, are the main drawbacks of Roche's method, although the read lengths in this technology have until recently been longer than in other methods making it especially suitable for *de novo* genome and transcriptome sequencing.

In the Illumina method reversible terminator nucleotides for the four bases each labelled with a different fluorescent dye are incorporated into the synthesised DNA strand. After incorporation into the DNA strand, the terminator nucleotide is detected and identified via its fluorescent dye, and contrary to the Roche technology, only one nucleotide can be added to the sequenced nucleotide chain at each cycle due to a blocking group present in the 3'-end of the base (Mardis, 2013). After the removal of the blocking group and the fluorescent dye, the synthesis cycle is repeated. Due to its cost-effectiveness, relatively long reads (300 bp with paired-end sequencing) and sufficient sequencing accuracy, the Illumina system is currently the most widely used NGS technology (Zhang et al., 2011).

Ion Torrent's (now part of Life Technologies) Personal Genome Machine (PGM) utilises an entirely different approach to the detection of an incorporated nucleotide. Instead of fluorescence and camera scanning, the PGM uses semiconductor technology to detect changes in the pH caused by the release of a proton at the incorporation of a complimentary base, and this has resulted in a smaller instrument size, shorter run times and lower sequencing costs (van Dijk et al., 2014). However, similar to Roche's technology, as several identical bases can be incorporated to the sequenced strand simultaneously, the correct detection of the number of identical bases in the homopolymer stretches is difficult (Buermans and den Dunnen, 2014). Initially the throughput of the PGM was considerably lower than in the competing technologies, but it has steadily increased.

Pacific Biosciences' SMRT sequencing is a single-molecule sequencing technology that measures the passage of a DNA sequence through a nanopore. It does not require amplification of the sequenced sample, but the method is sensitive enough to detect the incorporation of individual nucleotides and their base modifications. It also detects the sequence in real time making it a third generation sequencing technology. During the library preparation the sequenced sample fragments are made circular and in the pre-preparation of the sequencing template a single DNA polymerase is bound to the template (Buermans and den Dunnen, 2014). The sequencing machine detects the base-specific fluorescence of the incorporation of a phosphate-labelled nucleotide originating from the natural DNA synthesis of a single DNA polymerase in real time (van Dijk et al., 2014). Due to the circularity of the template, the sequenced molecule can be read from one to several times depending on the insert size and read length. The read lengths in the PacBio RS II machine are longer than in the other sequencing technologies, and read lengths of over 20 kb are regularly sampled.

In all of the technologies the sequencing step is done in parallel so that even hundreds of millions of sequence fragments are sequenced in one run. The current sequencing capacity of the Illumina HiSeq4000 machine is up to 1500 Gb, the capacity of Ion Torrent's PGM machine with Proton-I chip is up to 10 Gb and the capacity of the Pacific Biosciences' PacBio RS II machine is up to 5 Gb.

When the NGS technologies emerged, the read lengths were generally quite short, only around 30 bp, which limited their use in applications such as *de novo* genome sequencing. During the recent years both the read length and the throughput have increased steadily, and currently the typical read lengths range from 50 bp to several hundred base pairs depending on the sequencing application. The development of paired-end (Korbel et al., 2007, Ng et al., 2005, Ng et al., 2006) and mate-pair sequencing (Shendure et al., 2005) protocols, where the DNA fragment is sequenced from both ends instead of from just one end, and innovations such as strand-specific sequencing, have enabled the use of NGS for many applications, such as alternative splicing analysis, fusion transcript detection and detection of genomic structural variations (Fullwood et al., 2009, Campbell et al., 2008).

While the read length, sequencing platform and library type (single end or paired-end) affect the usability of the sequencing data, other project characteristics such as sequencing depth are important factors in a study. Sequencing depth, or coverage, refers to how many times any single genetic locus within the parental genome or transcriptome has been sequenced, i.e. in how many different, overlapping reads that nucleotide is present. The higher the number of reads in which the nucleotide is present, the higher the coverage or deeper the sequencing. The required sequencing depth typically depends on the aim of the study. For gene expression studies a relatively low sequencing depth may be sufficient to profile candidate genes. The detection of rare alternative splicing events, the *de novo* sequencing of complex genomes and the investigation of clonal populations during tumour development

requires a very much higher sequencing coverage to ensure a reliable and sensitive result.

Several different NGS applications have been developed that can be applied to provide content and context for different research questions. Whole genome re-sequencing (WGS) is the sequencing of the complete genome of an organism to a given depth for purposes of discovering genetic variants, changes in gene copy number, heterozygosity and potentially genomic aberrations (Wheeler et al., 2008, Bentley et al., 2008). WGS studies are performed with the understanding that a reference genome sequence is available; this influences the bioinformatics strategy that will be applied to the data, the depth to which the genome is sequenced and the length of the sequence reads that are determined to be sufficient. In targeted re-sequencing only a subset of the genome is sequenced, e.g. all of the exons (exome sequencing) or certain gene regions known to contribute to e.g. the genetic susceptibility to a disease within e.g. amplicon sequencing (Bilgüvar et al., 2010, Nguyen-Dumont et al., 2015). *De novo* sequencing is the sequencing of a genome or a transcriptome from an organism where no reference genome sequence is available (Santure et al., 2011, Guo et al., 2011) and it is expected that the DNA sequences obtained will be computationally assembled into contiguous fragments. Transcriptome sequencing, or RNA-seq, is a popular method for studying differential gene expression by comparing the relative abundance of transcripts between different experimental conditions (Mortazavi et al., 2008, Nagalakshmi et al., 2008). RNA-seq can also be used to identify alternatively spliced gene isoforms or long non-coding RNAs (Morin et al., 2008a, Iyer et al., 2015). Chromatin immunoprecipitation sequencing (ChIP-seq) is used to identify e.g. transcription factor binding sites and chromatin modifications within the genome (Barski et al., 2007, Lefrançois et al., 2009, Johnson et al., 2007, Robertson et al., 2007). NGS can also be used for sequencing of small non-coding RNAs and microRNAs (Creighton et al., 2009, Morin et al., 2008b), sequencing of methylated DNA regions (Lister et al., 2008, Meissner et al., 2008) and sequencing of metagenomic samples either through shotgun sequencing of random genomic DNA or through deep sequencing of ribosomal RNA sequences (Gilbert et al., 2008). The applications listed here are some of the most well-known and popular NGS applications and several methods specific for a certain sample type or experimental setup have been developed.

2.2.1 *Bioinformatics in NGS*

Due to the large amount of data produced by the next-generation sequencing machines, the data analysis is typically computationally intensive and requires significant computing resources in order to be performed in reasonable time. Several specialised bioinformatics approaches and tools have been developed to facilitate the analysis of NGS data, many of which are freely available as open-source software. The key analysis steps for some of the most popular NGS applications are briefly reviewed in this section.

For genome studies in organisms with substantial amounts of reference DNA sequence available, the first step in the analysis of NGS short sequence reads is the mapping of reads to the reference genome. This step is common across many NGS applications. During mapping computational heuristics are applied to pinpoint the location in the reference genome from which the sequence read is most likely to have come. Since individuals typically differ from the reference sequence by as much as 2% of base positions the heuristics are needed to accommodate a fuzziness. Several different mapping algorithms have been developed and many tools implementing these algorithms are freely available with typical best-of-breed methods including bwa, Bowtie, Tophat, STAR and subread (Langmead et al., 2009, Li and Durbin, 2009, Trapnell et al., 2009, Dobin et al., 2013, Liao et al., 2013). Some of the mappers, e.g. Tophat and STAR, are especially designed for eukaryotic RNA-seq data and can identify exon-exon junctions, which are not present in genomic DNA samples, but do appear in RNA samples, which include mature RNA molecules from which introns have been spliced. The different mapping tools have different strengths and their performance can vary depending on the type of data, as no mapping tool outperforms the others in every instance (Hatem et al., 2013). Thus the choice of the mapper should be based on the data type and the requirements of the experimental setup.

If no reference genome is available, the reads will need to be *de novo* assembled prior to further analysis. In *de novo* assembly the overlapping reads are used to identify longer consensus sequences, which ideally span the length of entire chromosomes, although in real-life eukaryotic studies this is rarely the case. These consensus sequences, which are called contigs, along with scaffolds, which are groups of contigs with gaps of known length between them, are the integral components of a genome assembly (Howison et al., 2013). For large and complex genomes, the sequencing coverage needs to be high and planned strategies that incorporate combinations of paired-end and mate-pair libraries of varying insert sizes across different sequencing platforms need to be used in combination in order to obtain accurate genome assemblies (Liu et al., 2014, Nystedt et al., 2013). Several software choices for *de novo* assembly are available, and the most commonly used ones can be divided into string-based and graph-based methods (Zhang et al 2011). The graph-based assemblers, especially assemblers using De Bruijn graph algorithms, are currently widely used, but with the longer read length of the third generation sequencers, traditional string-based methods may (once again) become more attractive than graph-based methods (Henson et al., 2012). Popular *de novo* genome assembly tools include e.g. MiRA, ABySS, ALLPATHS, SOAPdenovo and Velvet (Butler et al., 2008, Li et al., 2010, Zerbino and Birney, 2008, Simpson et al., 2009). Tools specialised in the assembly of transcriptomes from RNA-seq data have also been developed, e.g. Trinity (Grabherr et al., 2011) and SOAPdenovo-Trans (Xie et al., 2014). Assembly in particular can be very time consuming and computationally intensive, therefore the assembler should be chosen carefully.

In whole genome resequencing the aim is often to detect aberrations from the reference genome, which can include single nucleotide polymorphisms (SNPs),

insertions and deletions, copy number variations (CNVs) and chromosomal rearrangements. The GATK Genome analysis tool kit developed at the Broad Institute is a popular tool for identifying both SNPs and small insertions and deletions (McKenna et al., 2010). PEMer pipeline and Geometric analysis of structural variants tool on the other hand have been developed for the detection of larger structural variations, i.e. kilobase- to megabase-sized deletions, insertions, duplications and inversions (Korbel et al., 2009, Sindi et al., 2009). Additionally tools specialised in the annotation of detected SNPs are available, SnpEff (Cingolani et al., 2012) and Annovar (Wang et al., 2010) can for example identify the gene region where a SNP is located and the amino acid change in the coding sequence resulting from a SNP.

In ChIP-seq studies peak detection is usually performed subsequent to mapping. When the immunoprecipitated fragments are sequenced, the resulting reads form peak-like patterns on the regions where the studied molecule was bound. These peaks can be detected and thus the binding regions and patterns of the molecule identified. Peak detection algorithms include e.g. FindPeaks and MACS (Fejes et al., 2008, Zhang et al., 2008). Depending on the immunoprecipitated protein, the size and form of the peaks can differ considerably from the narrow peaks of transcription factors to the broad regions of histone marks, and different peak calling algorithms have been optimised for certain types of peaks (Bailey et al., 2013). Some peak calling tools can also perform a differential binding site analysis between conditions, as implemented in e.g. ChIPDiff (Xu et al., 2008). After peak detection the peak regions can be annotated and the binding motifs for the molecule in question can be identified with specialised tools like HOMER (Heinz et al., 2010).

After mapping gene level abundance estimations are usually performed in RNA-seq studies. The abundance estimations can be based on simply counting the amount of reads mapped per each gene, as implemented in HTSeq-count (Anders et al., 2014), or on more complex algorithms as in RSEM, which estimates the abundance by an iterative process called expectation maximisation (Li and Dewey, 2011). Also alignment-free methods for counting gene abundances are available, e.g. Sailfish, which uses a k-mer detection algorithm to calculate the relative abundances of genes within an RNA-seq data set (Patro et al., 2014). RNA-seq data can also be used to identify and quantify alternative transcripts, but this requires deep sequencing coverage and improvements in the analysis methods are still required (Consortium, 2014). Following the abundance estimations differential expression of genes between different conditions using statistical testing is performed. Many tools for the detection of differential expression have been developed and they are based on e.g. negative binomial models as implemented in edgeR (McCarthy et al., 2012) and DESeq2 (Love et al., 2014), or voom transformation followed by linear modelling as implemented in limma (Ritchie et al., 2015). The available statistical analysis tools for the detection of differentially expressed genes can exhibit significant differences depending on e.g. the number of replicates and the heterogeneity of the data, making no single method optimal for all occasions (Seyednasrollah et al., 2015).

Another analysis step common to most, if not all, NGS applications is annotation. Annotation brings biological meaning into the results and is fundamental to drawing coherent conclusions from the data. For organisms with a well-established reference genome this task is usually trivial, but for organisms without a reference genome, the annotation process is often difficult, and can range from heterologous transfer of information from public data repositories to *ab initio* prediction of genes and proteins (Yandell and Ence, 2012). Eventually most NGS analysis strategies converge into biology-oriented downstream analyses, which involve identification of e.g. pathways, cis-regulatory modules and regulatory networks and subsequently also the integration of prior information from different public databases (Werner, 2010).

2.2.2 *NGS in non-model organism research*

NGS has greatly facilitated the research on non-model organisms, as it doesn't require any previous knowledge on the organism's genome or transcriptome sequences. As the cost of NGS has steadily declined *de novo* sequencing of non-model organisms is now also possible even for individual research groups without huge budgets, and hence several non-model organism transcriptomes (Feldmeyer et al., 2011, Franchini et al., 2011, Shi et al., 2011, Fraser et al., 2011) and genomes (Diguistini et al., 2009, Wang et al., 2014a, Jiang et al., 2011) have been published in the recent years. Additionally, NGS has been used in non-model organism research to e.g. detect SNPs (Novaes et al., 2008, Iorizzo et al., 2011).

As a reference genome is rarely available for non-model organisms, and therefore a *de novo* assembly is usually required, sequencing technologies that provide long read lengths, such as Pacific Biosciences' SMRT sequencing, would be better suited for these types of sequencing projects, as they have the potential to span entire repeat regions and complete gene lengths (Koren et al., 2012). However, due to the considerably lower yield of sequenced bases and the higher cost of the sequencing, SMRT sequencing can also be used to complement shorter reads from other sequencing technologies to improve assembly quality (English et al., 2012).

NGS technologies have particularly enabled the sequencing of non-model organism transcriptomes. Transcript sequences are simpler to assemble than genomic sequences mainly due to the varying abundance of transcripts within an organism's mRNA pool resulting in a higher coverage of reads for highly expressed genes and to the lack of highly repetitive elements compared to the genomic sequences (Gibbons et al., 2009). In non-model organisms, transcriptome sequencing is used not only to study differential gene expression between different conditions (Mamidala et al., 2012, Feng et al., 2012), but also to characterise transcriptomes by gathering a representation of the genes of a species of interest (Sui et al., 2011, Santure et al., 2011).

3 AIMS OF THE STUDY

The aim of this doctoral work was to study lichen transcriptomics and genomics, and to investigate the molecular mechanisms behind lichen desiccation tolerance. Additionally the aim was to optimise high-throughput transcriptomics and genomics methods for lichen research and generate high-throughput sequence data for our target of choice, *Cladonia rangiferina*, in order to increase the publicly available lichen sequence data, which is still quite scarce.

Firstly we needed to compare and optimise different available RNA extraction methods for use in cDNA library construction, as lichens do not contain large amounts of RNA, but do contain several secondary metabolites, which inhibit the extraction of high-quality RNA.

The second aim was to obtain sequence data from wet lichen tissue using high-throughput next-generation sequencing and the more traditional Sanger EST sequencing. We were able to characterise the transcriptome of an un-stressed lichen and the sequences produced were utilised in the design of a custom microarray.

The third aim was to study the molecular mechanisms behind lichen desiccation tolerance in more detail using a custom microarray. RNA was extracted from lichen tissues in different stages of dehydration and rehydration and hybridised to the microarray to detect the most differentially expressed genes during the process of desiccation and rehydration.

Additionally we produced genomic DNA sequences from the thallus of *C. rangiferina* using next-generation sequencing. These sequences were *de novo* assembled and the genes within the assembled contigs were predicted. Finally the gene content was annotated and characterised.

4 MATERIALS AND METHODS

4.1 Material

Cladonia rangiferina was chosen as our model organism for its abundance in southern Finland. Thallus tissue used in the experiments was collected from the island of Kuusisto in Kaarina, Finland. After collection, it was cleaned and stored in desiccated state at -20 °C.

When wet lichen tissue was used for sample preparation, the tissue was wetted overnight with tap water prior to nucleic acid extraction (I, II). In III samples were taken in several different stages of rehydration and dehydration, and are summarised in Figure 2 and Table 1. Lichen tissue was rewetted using tap water and samples were collected at 15 minutes, 30 minutes, 1 hour and 3 hours into rehydration. The sample collected at 3 hours into rehydration was considered a wet lichen sample. The wet lichen tissue was left to desiccate at room temperature under normal laboratory conditions and samples were taken at 1 hour, 3 hour, 6 hour and 24 hour time-points during the desiccation process. The sample collected at 24 hours into desiccation was considered a dry lichen sample. The weight of the lichen tissue at all collection points during rehydration and dehydration was measured for the calculation of relative water content (RWC) values (III). 24 hours was selected as the final sample time-point because beyond this point no further changes in lichen tissue weight and RWC were observed. For the extraction of genomic DNA stored dry lichen tissue was used as the starting material (IV).

Table 1. The sample groups in III and their abbreviations used in the text.

Sample group name	Condition
W15m	Wetted for 15 min
W30m	Wetted for 30 min
W1h	Wetted for 1 h
Wet	Wetted for 3 h
D1h	Air-dried for 1 h
D3h	Air-dried for 3 h
D6h	Air-dried for 6 h
Dry	Air-dried for 24 h

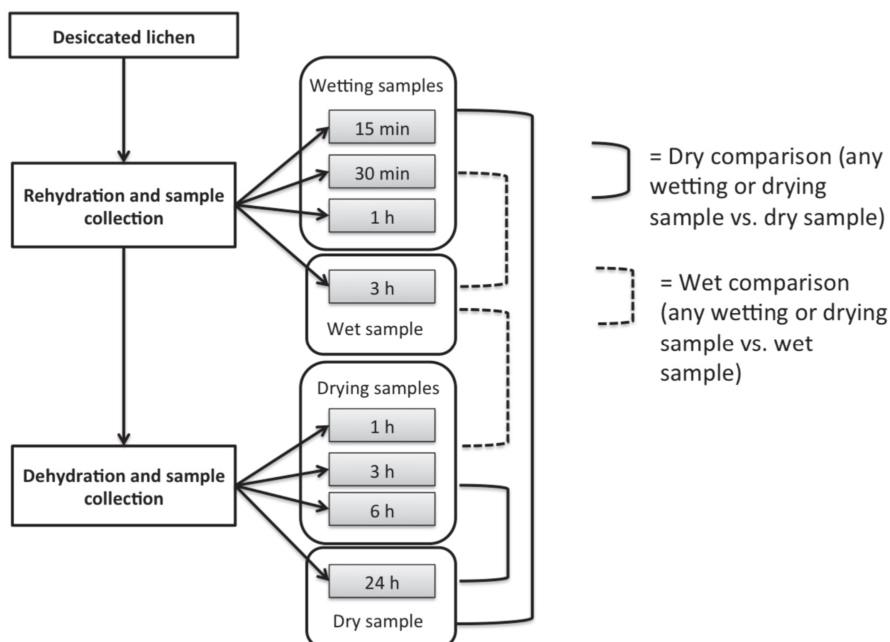

Figure 2. The microarray experimental procedure and sample set up. A flowchart illustrating sample set up, the naming of the samples, and the different comparisons between the sample groups in III.

The symbiotic partners (*C. rangiferina* and *Asterochloris* sp.) were cultured axenically using the modified Yamamoto method (II, III, IV) (Yamamoto, 1990). *Cladonia rangiferina* was cultured on Malt Yeast Extract (Yamamoto, 1990) agar plates and *Asterochloris* sp. on Organic Nutrient Medium for *Trebouxia* (Ahmadjian, 1967) agar plates at 21 °C. The mycobiont and photobiont material for nucleic acid extraction was collected from these culture plates. The identity of the axenically grown symbionts was confirmed by sequencing the ITS regions using ITS1F and ITS4F primers for the mycobiont and ITS1T and ITS4T primers for the photobiont (Gardes and Bruns, 1993, White et al., 1990).

All samples were flash frozen in liquid nitrogen and were ground to a fine powder using mortar and pestle (I-IV). The ground lichen or symbiont material was then used as starting material for the nucleic acid extractions.

4.2 Nucleic acid extractions

Several RNA extraction methods were compared and optimised to identify the method best suited for obtaining the required amounts of high-quality RNA for cDNA library construction (I). The evaluated methods included the Dong & Dunstan protocol

(Dong and Dunstan, 1996), the CTAB protocol (Gooding et al., 2001) combined with RNeasy Midi Kit (Qiagen, Germany), the Spectrum Plant Total RNA kit (Sigma Aldrich, Germany) and the TRIzol reagent (Invitrogen, USA).

The Dong & Dunstan protocol was modified by replacing the LiCl precipitation of RNA step with an ethanol precipitation of nucleic acids at -80 °C for 2 hours. 1 g of lichen tissue was added to 15 ml of extraction buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM EDTA, 2% SDS, 0.5% polyvinylpyrrolidone MW 360 000) added with 0.5 mM aurintricarboxylic acid and 14.3 mM β-mercaptoethanol. After a 10-minute incubation at 65 °C and centrifugation, 0.7 ml of 3 M potassium acetate (pH 4.8) was added to the supernatant followed by incubation on ice for 30 minutes and centrifugation. The supernatant was precipitated with ethanol, dissolved in water and extracted once with phenol and twice with phenol-chloroform-isoamylalcohol (24:23:1). The RNA was precipitated with ethanol and the dried pellet was dissolved in water.

The CTAB protocol was modified from Gooding et al. (2001), who had modified a pine tree RNA extraction method (Chang et al., 1993). 1 g of lichen tissue was added to 10 ml of pre-warmed (65 °C) extraction buffer (2% CTAB, 2% polyvinylpyrrolidone MW 40 000, 200 mM Tri-HCl pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g/l spermidine) added with 2% β-mercaptoethanol. The mixture was extracted twice using phenol-chloroform (1:1) and the supernatant was added to an equal volume of NTES buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and chloroform (1:1). The RNA was precipitated with ethanol and the dried pellet was dissolved in water. The extracted total RNA was cleaned using the RNeasy Midi Kit according to the manufacturer's instructions. Approximately 20 samples were pooled into one clean-up column.

When using the Spectrum Plant Total RNA kit for RNA extraction (I, II, III), 100 mg of lichen tissue was used as starting material and the extraction was performed according to the manufacturer’s instructions. For TRIzol reagent, the extraction procedure was modified from the manufacturer's instructions by adding an additional chloroform extraction step after the first chloroform extraction and replacing the isopropanol precipitation with an ethanol precipitation at -80 °C for 2 hours. Different lichen tissue/TRIzol reagent ratios were evaluated with 1 g of lichen tissue in a 15 ml volume of TRIzol reagent being optimal in terms of RNA yield and quality.

For cDNA library construction and NGS library preparation large amounts of total RNA were needed as starting material, and we therefore used the combination of an optimised CTAB extraction method and a commercial clean-up step in order to obtain high-quality RNA in a time- and cost-efficient manner (I, II). For microarray hybridisations a smaller amount of starting material was sufficient making the use of the Spectrum Plant Total RNA kit suitable (III).

mRNA was isolated from the total RNA with Nucleo-Trap mRNA kit (Macherey-Nagel, Duren, Germany) (I, II). Total genomic DNA was extracted from ground tissue

with Qiagen's Plant Mini Kit according to the manufacturer's instructions (IV) (Qiagen, Germany).

4.3 Nucleic acid quality control

The quality of the extracted nucleic acids was assessed using NanoDrop-1000 instrument (NanoDrop Technologies, USA) using the $A_{260/280}$ and $A_{260/230}$ ratios (I-IV). The quality of the extracted nucleic acid was also inspected visually following gel electrophoresis (I, II). The nucleic acids were resolved on 1.2% gels in TBE buffer at 80V for as long as it took for the marker dye to migrate to the end of the gel. Quality was evaluated by the uniformity of the smear, clear ribosomal RNA bands and the amount of high molecular weight material that likely corresponds to genomic or mitochondrial DNA (I, II). The quality of the samples hybridised on the custom microarray was additionally assessed using Bioanalyzer capillary electrophoresis instrument (III) (Agilent Technologies, USA). All of the samples hybridised to the microarray had a RNA Integrity Number (RIN) (Schroeder et al., 2006) value above 9.

4.4 cDNA library construction and EST sequencing

A phage cDNA library was constructed from the extracted mRNA using the ZAP-cDNA® Gigapack® III Gold Cloning (#200450) cDNA library synthesis kit (Stratagene, La Jolla, USA) according to manufacturer's instructions (I, II). Size fractionation was achieved using gel electrophoresis. DNA ladder fragments (Fermentas, Lithuania) were included in spare lanes during the electrophoresis to provide a reference as to expected molecular fragment sizes at different position on the gel. Gel slices corresponding to cDNA fragments of between 500-1,000 bp and 1,000-3,000 bp in size were excised and purified with QIAquick Gel Extraction kit (Qiagen, Germany). These fragment sizes were chosen based on previous experiments, which had shown that these fragment sizes ligate efficiently to the cloning vector (results not shown). The gel-purified cDNA was cloned into a phage library.

In order to obtain EST sequences from both the lichen tissue and the axenically grown symbionts (I-IV), cDNA library clones were sequenced on an ABI PRISM 3130xl Genetic Analyzer capillary DNA sequencer (Life Technologies, USA) following a BigDye v3.1 labeling reaction.

4.5 Next-generation sequencing

The double-stranded cDNA for the transcriptome next-generation sequencing (II) was prepared using SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen, Life Technologies, USA) according to manufacturer's instructions. Invitrogen's Oligo $dT_{(12–18)}$ primer was used in the synthesis reaction. The concentration of the cDNA was measured with NanoDrop and altogether 5.12 µg of double-stranded cDNA was used as starting material for the Roche GS FLX sequencing (II). The transcriptome high-

throughput sequencing was performed at the DNA Sequencing and Genomics Laboratory at the Institute of Biotechnology at University of Helsinki, Helsinki, Finland according to previously described methods (Margulies et al., 2005). The cDNA was amplified with Phi29 polymerase (GenomePhi, GE Healthcare, USA) and a single-stranded sequencing library was created according to the FLX instructions. The GS FLX run data were filtered with the GS Run Browser and reads not passing the quality filters were removed from the raw data.

For the genome sequencing (IV) altogether 21.9 μg of genomic DNA was extracted and used as starting material for the library preparation. Paired-end library was constructed of the lichen DNA using Illumina's kits and instructions (Illumina Inc., USA). The library was sequenced on two lanes in Illumina's HiSeq2000 sequencing instrument with paired-end protocol and 100 bp read length. The base calling and initial quality filtering were performed with Illumina's software. The genome sequencing (IV) was performed at the MGRC, Kuala Lumpur, Malaysia.

4.6 Microarray design and hybridisations

The lichen custom microarray (III) was manufactured according to the Agilent 4x44K array format and oligonucleotide probes were designed and optimised using the manufacturer's eArray tool (https://earray.chem.agilent.com/earray/). Transcriptome NGS reads from the Roche GS FLX run and EST sequences derived from lichen tissue were used as substrate for the array design process. The Roche GS FLX reads were assembled with MIRA2 (Chevreux et al., 2004) prior to the microarray probe design. The sequences were split into two groups according to the contig lengths. Sequences of less than 450 nt in length were classified as short sequences while sequences longer than or equal to 450 nt in length were classified as long. For the 20,779 short sequences a single 60 nt probe per target sequence was designed while for the 10,676 long sequences two 60 nt probes per target sequence were designed. This resulted in altogether 20,663 probes for the short sequences and 20,071 probes for the long sequences. In addition a replicate probe group of 19 probes was created as recommended by Agilent, and the probes within the replicate probe group were each replicated ten times on the array. Agilent control probes were also included into the array design.

The RNA sample labeling and hybridisation for the microarray hybridisations (III) were performed using the manufacturer's One-Color Microarray-Based Gene Expression Analysis protocol (Agilent, USA, Version 5.7). 600 ng of total RNA was amplified and labeled with Cy3 using the Quick Amp Labeling kit (Agilent, USA). The samples were processed with an exogenous control sample provided through an RNA Spike-in kit (Agilent, USA). 1.65 μg of Cy3-labelled sample was hybridised to the 4x44K custom array in 65 °C overnight using the provided Gene Expression Hybridization kit. The arrays were washed using the Gene Expression Wash Pack and Stabilization and Drying solutions also provided in the custom DNA microarray gene expression profiling kit. The microarray hybridisations and scans were performed at

the Finnish Microarray and Sequencing Centre at the Centre for Biotechnology in Turku, Finland.

The DNA microarrays (III) were scanned using an Agilent Technologies' Scanner model G2565CA. The expression data were derived from the image files using Agilent's Feature Extraction software, version 10.5.1.1, using grid 024161_D_F_20090605, protocol GE1_105_Dec08 and QC metric set GE1_QCMT_Dec08. These microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE47624 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47624).

4.7 Quantitative real-time RT-PCR validations

Eleven transcripts (*cr_lrc491*, *cr_DMini_2305E01*, *cr_lrc5, cr_c3825*, *cr_c4168*, *cr_DMini_673H04*, *cr_lrc323*, *cr_c10766*, *cr_c15269*, *cr_lrc282*, and *cr_c18326*) were selected for quantitative RT-PCR validation of the microarray results (III). The transcript identified as *cr_lrc491* was chosen as the endogenous control because it demonstrated similar signal-intensity values across all samples. Five other transcripts (*cr_DMini_2305E01*, *cr_lrc5, cr_c3825*, *cr_c4168*, and *cr_DMini_673H04*) also had steady intensity values across the samples, whereas transcripts *cr_lrc323*, *cr_c10766*, *cr_c15269*, and *cr_lrc282* showed different expression levels between the samples.

The primers were designed using Roche's Universal Probe Library with the Probe Finder software version 2.45 (http://qpcr.probefinder.com/organism.jsp). An optimal real-time PCR assay was successfully designed for all of the selected transcripts, and the primers were manufactured by Oligomer Ltd (Helsinki, Finland). 1 µg of RNA was reverse transcribed into cDNA using Dynazyme reverse transcriptase (Finnzymes, Finland). Quantitative RT-PCR reactions were performed for each sample in four replicates using KAPA qPCR Master Mix (Kapa Biosystems, USA) with the following amplification protocol: 10 minutes at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, 10 s at 8 °C. The transcript identified as *cr_lrc491* was used as an endogenous control and fold change values compared to the Dry sample were calculated using the RQ Manager v.1.2 software (Life Technologies, USA).

4.8 Bioinformatics and data analyses

Base calling for the Sanger sequenced EST sequences derived from lichen tissue and axenically grown symbionts (I-IV) was performed using *phred* (Ewing et al., 1998). The EST sequences were compared against a modified NCBI *UniVec* database using *cross_match* to identify vector and polylinker sequence substrings.

The transcriptome *de novo* assembly (II) was performed using CLC Genomics Workbench software version 4.9 (CLCBio, Denmark). Prior to the assembly the NGS reads with low quality bases were trimmed and the adapter sequences potentially

present at either end of the GS FLX sequences were removed in the CLC Genomics Workbench. Sequences shorter than 15 nt were removed from the analysis. In the transcriptome *de novo* assembly (II) the minimum contig length was set to 250 bp and voting method was chosen as the conflict resolution parameter. The transcriptome sequence reads were then mapped back to the assembled contigs within the CLC Genomics Workbench assembly workflow.

The genome *de novo* assembly (IV) was performed with velvet version 1.2.10 using a k-mer size of 63 (Zerbino and Birney, 2008). Prior to the assembly the quality of the sequences was controlled with FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and based on these results the last 20 nucleotides from read 1 and the last 25 nucleotides from read 2 were trimmed with prepare_reads.pl script available in the velvet installation. The N50 value of an assembly is calculated by summarising the lengths of the biggest contigs until 50% of the total genome or transcriptome length is reached. The minimum contig length in this set is the number that is used to report the N50 value of a *de novo* assembly (II, IV).

Eclat (Friedel et al., 2005) was used to classify the lichen sequences as of fungal or algal origin (II-IV). The EST sequences obtained from the axenically grown algal and fungal symbiont cDNA libraries were used to train *Eclat* and build a model file for the classification. The minimum sequence length for classification was set at 100 bp.

CEGMA (Parra et al., 2007) was used to identify conserved core eukaryotic genes present in the *de novo* genome assembly (IV). Gene prediction from the assembled contigs was performed using *geneid* (Guigó, 1998) and *Augustus* (Stanke et al., 2004). Candidate genes encoding for PKS and NRPS genes were identified using SMURF (Khaldi et al., 2010). BLASTX (Altschul et al., 1990) was used to compare the obtained sequences to the NCBI non-redundant (nr) protein sequence database (Benson et al., 2008) (I-IV). BLASTX matches were filtered using an arbitrary cutoff of 1e-10 (I, II), 1e-6 (IV) or 1e-3 (III). The Blast2GO tool (Conesa et al., 2005) was used to analyse the BLASTX homology search results. This analysis was used to assign candidate GO (Ashburner et al., 2000) and enzyme code (EC) annotations, to perform Interpro scans and to identify candidate protein sequence domains and to map sequences onto KEGG pathways (II-IV). Combined graphs were produced from the GO annotation results and the most enriched GO terms were visualised in multilevel format.

The microarray raw data (III) were analysed using R and Bioconductor (Gentleman et al., 2004, Team, 2008). Quantile normalisation methods implemented in the Limma package (Smyth, 2004) were used to reduce technical variability. Several different visualisation methods were used to control for the quality of the data. Each time point during dehydration and rehydration was compared to both the Wet and Dry sample to identify the differentially expressed genes in each of these comparisons (Figure 2). The Limma package, which applies linear modeling with a modified t-test, was used

for the statistical testing and the thresholds for the up- and down-regulated genes were chosen individually for each comparison.

Each sample group comparison in the microarray experiment (III) was tested for enrichment of GO terms using the Fisher's Exact Test method as implemented in Blast2GO. A fold-change threshold of > |1.5| and a p-value threshold of 0.01 were assigned for determination of sensitivity for the Fisher's Exact Test. A one-tailed test with p-value threshold of 0.05 was used to distinguish the over-represented GO terms in each sample group comparison.

The clustering of the transcript expression values (III) and the plotting of their heat maps was performed using the GENE-E tool (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). Euclidean clustering was selected for the clustering method and average linkage as the linkage method. Logarithmic expression values were used in the input data.

5 RESULTS AND DISCUSSION

This doctoral work consisted of characterising the *Cladonia rangiferina* transcriptome and genome and studying the gene expression of *C. rangiferina* during different stages of desiccation and subsequent rehydration. A summary of the results of the original publications is presented here:

5.1 Optimising RNA extraction methods for lichen samples (I)

Lichen thalli are known to contain large amounts of secondary metabolites such as polyphenols (Palmqvist, 2000). These compounds can interfere with RNA isolation protocols lowering the yield and quality of the extracted RNA (Logemann et al., 1987), thus making the extraction of large amounts of RNA challenging. High-quality RNA is the prerequisite for all transcriptome studies regardless of the analysis method, and therefore different RNA extraction strategies were compared to study their usefulness with lichen samples.

Five different RNA extraction methods were evaluated: Spectrum Plant Total RNA kit, TRIzol reagent, Dong & Dunstan method, CTAB method, and CTAB combined with RNeasy Midi Kit. The conventional RNA extraction methods (TRIzol reagent, Dong & Dunstan method, and CTAB method) yielded large amounts of RNA, but the $A_{260/230}$ ratios were low. This indicates the presence of polyphenols and other secondary metabolites. However, the amount of starting material can be scaled up with these methods allowing for the extraction of relatively large quantities of impure total RNA, which can then be further purified with a commercial purification kit. The commercial Spectrum Plant Total RNA kit yielded high-quality RNA, but the amount of starting material is limited to 100 mg, rendering the method time-consuming and expensive for applications such as conventional phage cDNA library construction, which require large amounts of total RNA. However for technologies, which do not require such large amounts of total RNA as starting material the commercial Spectrum Plant Total RNA extraction kit is suitable (III). The CTAB method combined with a commercial RNA clean-up step was found to be the most suitable method in terms of RNA yield and quality, time and cost for applications requiring large amounts of total RNA. With this approach the $A_{260/280}$ and $A_{260/230}$ ratios of the extracted RNA were both >2, and this method was successfully used for RNA extraction for phage cDNA library construction (I, II) and next-generation sequencing library preparation (II).

5.2 Assembly and characterisation of *Cladonia rangiferina* transcriptome and fungal genome (II, IV)

To characterise the transcriptome and the fungal genome of *Cladonia rangiferina* high-throughput next-generation sequencing and traditional EST sequencing were performed. The EST sequences from Sanger sequencing and NGS reads generated

using the Roche GS FLX technology were used to characterise the *C. rangiferina* transcriptome (II) and to design a custom microarray for further studying the molecular mechanisms attributing to lichen desiccation tolerance (III). Paired-end reads sequenced with Illumina HiSeq2000 instrument, and derived from the lichen thallus, were used in the *de novo* fungal genome assembly (IV).

Altogether 240,990 high-quality reads were obtained from the whole transcriptome NGS sequencing run along with 2,990 EST sequences from traditional Sanger sequencing (II). These reads were assembled into 16,204 contigs and 49,587 singletons. The average length of the contigs was 528 nt with a maximum length of 5,426 nt and an N50 value of 569 nt. The average length of the singletons was 226 nt and the N50 value 231 nt. 79.7% of the reads were included in the assembled contigs while 20.3% of the reads remained as singletons. Similar contig and singleton values have been reported for other non-model organism *de novo* transcriptome assemblies (de la Torre et al., 2010, Sun et al., 2011).

The sequencing of the genomic DNA yielded in total 201,907,082 raw sequence reads (IV). After quality control, filtering and trimming 120,486,234 high-quality reads were included in the fungal genome *de novo* assembly, and it resulted in 1,378 contigs with an N50 value of 60,551 nt, maximum contig length of 206,467 nt and a total assembly length of 32,300,894 nt. 97,278,730 reads (80.7% of the included read amount) were used in the genome assembly. The total length of the assembly (32 Mb) is similar to that obtained from other lichen-forming fungal *de novo* assemblies (Park et al., 2014, Park et al., 2013b, Park et al., 2013a, Wang et al., 2014b). The main statistics of both the transcriptome and fungal genome assembly are presented in Table 2.

Table 2. The statistics of the transcriptome (II) and fungal genome assemblies (IV).

	Transcriptome assembly	Fungal genome assembly
Total number of reads	243,980	201,907,082
Number of reads used for assembly	243,980	120,486,234
Number of contigs	16,204	1,378
Maximum length	5,426	206,529
Minimum length	87	125
Median length	403	9,894
N50	569	60,551

To assess the quality of the fungal genome assembly, the presence of core conserved eukaryotic genes was assessed (IV). 234 genes out of 248 were discovered in the assembled contigs resulting in a 94.35% completeness. Thus, most of the conserved eukaryotic genes are present in the genome assembly, and the completeness of the proteins is similar to other lichen-forming fungal genome and lichen metagenome assemblies (McDonald et al., 2013). Prediction of coding regions in the assembled contigs resulted in 9,211 predicted genes, 8,855 of which were longer than 100 bp. Gene prediction was repeated also with another tool and similar results were obtained with 9,463 predicted genes. The lichen-forming fungi, *Endocarpon pusillum* and *Caloplaca flavorubescens*, have been previously reported to contain 9,285 and 9,695 genes, respectively (Park et al., 2013b, Wang et al., 2014b), which is similar to the amount of genes predicted in this fungal genome assembly. Additionally 42 PKS or PKS-like fungal genes and 6 NRPS or NRPS-like fungal genes were identified from the set of predicted genes, which is in concordance with the amount of identified PKS and PKS-like genes in another lichen-forming fungal species, *Cladonia metacorallifera* (Park et al., 2014).

Both the transcriptome and genome reads were obtained from *C. rangiferina* thallus and therefore contained sequences that originated from both the mycobiont and the photobiont. The EST sequences obtained from the axenically grown algal and fungal symbionts were used as training sets for the development of an *Eclat* classifier that could be used to predict the species of origin for the thallus-derived sequences. Classification of the assembled transcriptomic sequences and the predicted genes of the fungal genome assembly was performed. The classification of the contigs and singletons from the transcriptome assembly resulted in 62.8% of the sequences being classified as fungal and 37.2% as of algal origin (II). The contigs and singletons were also classified separately and then 78.4% of the contigs were classified as fungal and 21.6% as algal whereas only 57.4% of the singletons were classified as fungal and 42.6% as algal. Previously 7% of the cells in lichen symbiosis have been estimated as algal cells (Collins and Farrar, 1978) and in a more recent study 10% of proteins in *Lobaria pulmonaria* protein spectra were assigned to the green algal photobiont (Schneider et al., 2011). The classification results of the transcriptome sequences suggest a significantly higher ratio of algal transcripts present in the lichen symbiosis, but confirm the dominance of the fungal mycobiont in the lichen symbiosis also in the transcriptional level (II). The transcriptome survey however does not provide a robust quantitative assessment of the number of cells and it is plausible that the fewer algal cells may be transcriptionally more active than the structural fungal cells.

In the fungal genome assembly 98.7% of the predicted genes were classified as fungal and only 1.3% as algal origin (IV). Although we used DNA from the symbiotic lichen thallus as starting material in the sequencing, the similarity of the assembly to other assemblies of lichen-forming fungi and the low amount of genes classified as algal origin indicates that the assembly mainly includes reads derived from the mycobiont. About 80% of the filtered, high-quality reads were used in the assembly, thus the remaining 20% of the reads, which were not included in the assembly, could be comprised of reads derived from the algal symbiont and other non-fungal sources.

The assembly of reads derived from the lichen thallus can be more challenging, but we chose to sequence the intact lichen thallus as culturing of the lichen symbiotic partners axenically can be difficult and very time-consuming.

With a non-model organism annotation is often the bottleneck of the analysis. Especially with an organism like lichen, which has a very limited amount of sequences in the public databases, it is expected that a large proportion of the sequences cannot be assigned a proper annotation from the public data repositories. BLASTX analysis of the assembled contigs and singletons from the transcriptome sequencing (II) revealed that 34.4% of the sequences (22,662 sequences) had a match in the non-redundant (nr) database. For contigs alone this figure was higher, 57.2%. Other non-model organism transcriptome assemblies have similarly reported lower BLAST match percentages for singletons (Hsiao et al., 2011) and Joneson et al. (2011) found a significant homology to 50% of *Cladonia grayi* sequences in the nr database. Lowering the expectation value threshold yields higher match percentages, as 71.4% of the sequences used to design the lichen microarray probes had a match to the nr database with an E value cut off of 1e-3 (III). But 33.9% of these sequences had a match annotated only as “conserved hypothetical protein”, “hypothetical protein” or “predicted protein” resulting in 27.5% of the probe sequences having an unambiguous and natively annotated reference protein (III). However, when performing the BLASTX analysis for the predicted genes from the genome assembly (IV) a few years later, the percentage of sequences having a match in the nr database had increased substantially to 81.9%.

A majority of the lichen transcriptome sequences had a best BLAST match to either a fungus or an alga. 73.2% of the annotated transcriptome sequences had the best BLAST match to a fungal species, while 11.1% had the best match to an algal species, and 4.8% to a plant species (II). However, only 0.6% of the transcriptome sequences had a lichen species as the best BLAST match (II), whereas for the predicted genes from the fungal genome assembly lichen-forming fungi were the second biggest group with 8.4% of the hits (IV), indicating a substantial increase in lichen-derived sequence information in public databases in the intervening period of five years in which the analyses were performed. The majority of the BLAST hits (90.5%) for the predicted genes of the fungal genome assembly were to a fungal species (IV), adding to the conclusion that the assembly mainly includes reads derived from the mycobiont.

27.9% of the transcriptome sequences (II) and 81.8% of the predicted genes from the fungal genome assembly (IV) had a match in the InterPro database, and this suggests that although a reasonable proportion of the sequences contain a number of recognizable protein motifs, there are still many unrecognizable sequences, some of which may contain novel protein structures. In the genome assembly 106 genes with a cytochrome P450 domain were identified (IV), which is similar to other lichen-forming fungal genome *de novo* assemblies (Park et al., 2013b, Park et al., 2014), along with other important proteins and motifs such as transcriptome factors, polyketide synthases, dehydrogenases and protein kinases.

The GO and KEGG databases were used for the functional analysis of the sequences from both the transcriptome and genome assemblies. GO annotations are divided into three main categories: biological process (BP), cellular component (CC) and molecular function (MF). 22,736 contig and singleton sequences from the transcriptome sequencing were assigned to BP, 13,086 sequences to CC, and 29,170 to MF (II). The GO terms with the most associated sequences in BP category were oxidation reduction, RNA metabolic process, and catabolic process. In CC category the terms with the most associated sequences were cytoplasmic part, intracellular organelle part, and nucleus. In MF category the terms were ATP binding, nucleic acid binding, and oxidoreductase activity (II). 4,513 predicted genes (51%) from the genome assembly had a GO annotation, and the GO terms with most associated sequences in BP category were oxidation-reduction process, small molecule metabolic process, and gene expression, in CC category integral to membrane, cytoplasmic part, and nucleus, and in MF category oxidoreductase activity, nucleic acid binding, and protein binding (IV).

In the KEGG database genes are annotated to mainly metabolic and biochemical pathways. In the transcriptome assembly a total of 7,466 sequences were assigned to 60 identified KEGG pathways. The pathways with most associated sequences for the transcriptome contigs and singletons were purine metabolism, methane metabolism, and pyrimidine metabolism (II). In the fungal genome assembly, 1,070 predicted genes were associated with a KEGG pathway with altogether 115 identified KEGG pathways represented (IV). The KEGG pathways with the most associated sequences within the genome assembly were purine metabolism, thiamine metabolism, and pyrimidine metabolism. Many of the top GO terms were similar in the fungal genome assembly and the transcriptome assembly, especially for the cellular component and molecular function categories. Despite the increase in genes having a BLAST match and the increase in GO annotations in the genome assembly, the number of genes associated with each KEGG pathway is still very similar to the results in the transcriptome assembly. Also in the genome assembly only a small percentage of the genes were associated with a KEGG pathway, although the number of identified KEGG pathways increased significantly from the transcriptome assembly. However, this was quite expected, as lichen genomics is still in its infancy and therefore currently mainly the amount of recognised sequences has increased but the proper annotation of these sequences is still lacking.

A significant number of the transcriptome sequences were associated with GO terms related to oxidation. These terms could reflect the removal of ROS during normal metabolism, which has been observed in lichens (Kranner et al., 2008). High amounts of reduced glutathione have previously been measured in undesiccated lichens indicating an active constitutive protection mechanism against ROS (Kranner, 2002). The glutathione metabolism pathway was identified within this transcriptome assembly and could potentially be connected with the constitutive protection mechanisms.

Several enriched GO terms and most of the identified KEGG pathways within the transcriptome assembly were involved in energy, nucleotide or amino acid metabolic

processes (II), and pathways involved in photosynthesis were among the KEGG pathways with the highest number of sequences. These results are consistent with Schneider et al. (2011), who identified proteins involved in post-translational modifications, energy production and conversion as highly abundant in the mycobiont *Lobaria pulmonaria*, and proteins involved in energy production and conversion strongly dominating the protein fraction of the green algal photobiont *Dictyochloropsis reticulata* (Schneider et al., 2011).

The photosynthesising partner produces carbohydrates, which are leaked, taken up by the fungal partner and eventually converted to arabitol and mannitol through the phosphate pentose pathway (Lines et al., 1989). This mechanism is most likely active in the studied lichen thallus, and it could potentially be reflected by the many transport-related GO terms and the pentose phosphate pathway, which were identified within the transcriptome sequences (II). Surprisingly, methane metabolism was identified as one of the KEGG pathways with the biggest number associated sequences in the transcriptome assembly. These sequences could potentially be novel, lichen-specific sequences, which have a high homology to the proteins associated with methane metabolism, but which are in reality associated with an uncharacterised pathway, e.g. the production of a lichen-specific secondary metabolite.

5.3 Whole transcriptome characterisation of *Cladonia rangiferina* during desiccation and rehydration (III)

To study the molecular mechanisms underpinning lichen desiccation tolerance samples at different stages of rehydration and dehydration were collected and hybridised to a custom lichen microarray to analyse the differences in gene expression between the different sample groups. The sample groups and their abbreviations are summarised in Figure 2 and Table 1. The RWC values were calculated for the samples, and during wetting the RWC was 13% at 15 minutes, 30.9% at 30 minutes, 62.1% at one hour and 100% at three hours. The RWC of the samples during drying was 45.3% at one hour, 5.7% at three hours, 0% at six hours and 0% at 24 hours. The RWC values are likely to be higher than measured, as the lichen thallus was dried only in room temperature under normal laboratory conditions. The D6h and Dry samples have potentially obtained moisture from air humidity during the drying process, as we detected changes in gene expression for these samples despite the low RWC values.

The quality of the microarray data was assessed with several different methods, including hierarchical clustering, correlation analysis with Pearson's metrics and principal component analysis, to understand the sample relations and to identify possible outliers. The biological replicates within each sample group were highly reproducible with a minimum correlation value of 0.943. The correlation values of samples between different sample groups varied from 0.775 to 0.994, indicating that some sample groups differ from one another rather profoundly. We hypothesised the metabolic processes taking place in the lichen thallus during wetting and drying to be large, systemic changes, which would correspond to the lower correlation values

between samples from different sample groups and the high number of differentially expressed (DE) genes despite the strict filtering thresholds.

The microarray results were validated using quantitative RT-PCR. Three of the eleven designed assays did not produce any amplified product in the quantitative RT-PCR validations, but the remaining assay results corresponded well with microarray results. 91.1% of the measurements yielded similar results with both methods, although significant variation was detected between the samples in some sample groups in the quantitative RT-PCR analysis.

The thresholds for filtering the DE genes were chosen individually for each pairwise comparison. The filtering thresholds and the number of detected DE genes are presented in Table 3. The smallest number of DE genes detected with the thresholds (Table 3) was 162 genes in comparison W1h vs. Wet and the largest number of DE genes was 1,108 genes in comparison W15m vs. Dry. The ratio of up- and down-regulated genes varied considerably between the comparisons; in W1h vs. Wet comparison approximately half of the DE genes were up-regulated and half down-regulated, whereas in Wet vs. Dry comparison only 86 genes were down-regulated out of a total of 675 DE genes.

Table 3. Filtering parameters for all comparisons and the number of differentially expressed genes in III. FC = fold change, logFC = 2-logarithmic fold change, P = p value, FDR = false discovery rate, Total = total number of differently expressed genes, Up = number of up-regulated genes, Down = number of down-regulated genes.

Comparison	FC	logFC	P type	P	Total	Up	Down
W15m vs. Dry	32	5	FDR	0.001	1108	531	577
W15m vs. Wet	32	5	FDR	0.001	931	348	583
W30m vs. Dry	32	5	FDR	0.001	572	442	130
W30m vs. Wet	8	3	FDR	0.01	560	402	158
W1h vs. Dry	32	5	FDR	0.001	595	398	197
W1h vs. Wet	8	3	P value	0.001	162	80	82
Wet vs. Dry	16	4	FDR	0.001	675	589	86
D1h vs. Dry	16	4	FDR	0.001	980	599	381
D1h vs. Wet	16	4	FDR	0.001	919	393	526
D3h vs. Dry	32	5	FDR	0.001	645	430	215
D3h vs. Wet	32	5	FDR	0.001	614	266	348
D6h vs. Dry	32	5	FDR	0.001	663	462	201
D6h vs. Wet	32	5	FDR	0.001	624	317	307

The most differentially expressed genes in the microarray experiment across all of the studied samples did not show any clear sequence orthologues in the public sequence database or they show sequence similarity only to hypothetical proteins. In addition unannotated genes are enriched within the DE gene lists when compared to the whole data. For many sample groups close to 50% of the DE genes were

unannotated with the highest percentage being 66.3%. This could suggest that the genes most differentially expressed during dehydration and rehydration are lichen-specific or –adapted genes, or the sequences consist predominantly of UTRs and a known coding sequence has been missed. When more permissive thresholds were used for filtering of the DE genes, the percentage of unannotated genes was similar to the whole data, in which 38.6% of the probe sequences did not have a match in the nr database. Similarly in rehydrated *Tortula ruralis*, a desiccation-tolerant bryophyte, the most abundant transcripts did not match any known sequences (Oliver et al., 2004).

The most common annotated up-regulated genes within all of the comparisons were CaaX farnesyltransferase alpha subunit, U5 snRNP component, nuclear pore complex subunit Nup192, Swr1p complex component and cytochrome P450 family protein. The most common annotated down-regulated genes were heat shock protein HSP98, ion channel, nitrite reductase and siroheme synthase. The five most differentially expressed, annotated genes for each comparison are listed in Table 4. The W15m sample group shared only one of the most common DE genes with the other sample groups; the nuclear pore complex subunit Nup192 in up-regulated W15m vs. Dry comparison. However, the W15m vs. Wet and W15m vs. Dry comparisons had four of the five top annotated up-regulated DE genes and all top five annotated down-regulated DE genes in common.

The expression values of the 20 most DE genes with primary annotations from each comparison were clustered to study trends in gene expression in the different sample groups and to detect similarities in gene expression patterns among the sample groups. The W15m group was clearly different from all of the other sample groups, as was evident also from other analyses such as hierarchical clustering of the entire dataset and the top annotated DE genes (Table 4) suggesting that the molecular mechanisms active in the early stages of rehydration differ significantly from the latter stages and from dehydration. GO terms related to molecule transport and localisation were enriched in the W15m sample group indicating a potential activation of these responses at the onset of rehydration.

According to our results, the differential expression of short chain dehydrogenase and alcohol dehydrogenase continues past the initial rehydration. Alcohol dehydrogenases take part in detoxification reactions (Leterrier et al., 2011), and therefore their rehydration-induced up-regulation could be due to the detoxification of ROS and other harmful metabolites accumulated during desiccation. In previous studies alcohol dehydrogenase deficient tomatoes (Senthil-Kumar et al., 2010) and *Arabidopsis thaliana* plants (Conley et al., 1999) have exhibited increased susceptibility to osmotic stress. The role of alcohol dehydrogenase family proteins within the polyol synthesis pathway could contribute towards protection against water deficiency (Collett et al., 2004). The W15m and W30m sample groups also had some enriched GO terms in common, most of which were involved in molecular transport. Transcripts encoding PSII proteins are known to be stored during drying in desiccation-tolerant plants (Collett et al., 2003) to minimise the time needed to restart growth upon rehydration, thus the GO terms involved in molecule transport could

Table 4. The top five annotated genes for each comparison in III. Up = up-regulated genes, down = down-regulated genes, A = algal sequence, F = fungal sequence.

	Wetting sample groups vs. Wet					
	Up W15m vs. Wet	Down W15m vs. Wet	Up W30m vs. Wet	Down W30m vs. Wet	Up W1h vs. Wet	Down W1h vs. Wet
Annotation	Short-chain dehydrogenase, putative, F	Aspartic-type endopeptidase, putative, F	Short-chain dehydrogenase, putative, F	Nitrite reductase, F	CaaX farnesyltransferase alpha subunit, F	Nitrite reductase, F
	Mitochondrial molecular chaperone, F	Enoyl-acyl-carrier-proteinreductase 1, F	CaaX farnesyltransferase alpha subunit, F	30 kDa heat shock protein, F	Beta-glucosidase, putative, F	30 kDa heat shock protein, F
	Alcohol dehydrogenase, F	NEDD8 conjugating enzyme, F	Mitochondrial molecular chaperone, F	Ion channel, F	Non-classical export protein Nce102, putative, F	Siroheme synthase, putative, F
	PaaI thioesterase family protein, putative, F	DNA-directed RNA polymerase II subunit, F	Beta-glucosidase, putative, F	Hsp98/Hsp104/ClpA, putative, F	40S ribosomal protein, A	Ion channel, F
	Membrane-spanning ATPase, F	Eukaryotic translation initiation factor 3, F	Alcohol dehydrogenase, F	Hsp98, F	Vesicle coat complex COPII, subunit Sec24 family protein, F	DNA binding protein SART-1, F

	Wetting sample groups vs. Dry							
	Up W15m vs. Dry	Down W15m vs. Dry	Up W30m vs. Dry	Down W30m vs. Dry	Up W1h vs. Dry	Down W1h vs. Dry	Up Wet vs. Dry	Down Wet vs. Dry
Annotation	Short-chain dehydrogenase, putative, F	Enoyl-acyl-carrier-proteinreductase 1, F	Nuclear pore complex subunit Nup192, putative, F	Ion channel, F	Nuclear pore, complex Nup192, putative, F	Ion channel, F	DNA-directed RNA polymerase, F	Ion channel, F
	Nuclear pore complex subunit Nup192, putative, F	Aspartic-type endopeptidase, putative, F	Riboflavin aldehyde-forming enzyme, F	Nitrite reductase, F	Swr1p complex component, F	Nitrite reductase, F	Cytochrome P450 family protein, F	Imidazole glycerol phosphate synthase, A
	Mitochondrial molecular chaperone, F	NEDD8 conjugating enzyme, F	DNA-directed RNA polymerase, F	30 kDa heat shock protein, F	Riboflavin aldehyde-forming enzyme, F	Siroheme synthase, putative, F	Karyopherin, F	26S protease regulatory subunit, A
	PaaI thioesterase family protein, putative, F	Eukaryotic translation initiation factor 3, F	Cytochrome P450 family protein, F	Siroheme synthase, putative, F	Cytochrome P450 family protein, F	30 kDa heat shock protein, F	Swr1p complex component, F	40S ribosomal protein, A
	Alcohol dehydrogenase, F	DNA-directed RNA polymerase II subunit, F	Swr1p complex component, F	Copia-type polyprotein, F	DDHD domain protein, F	Imidazole glycerol phosphate synthase, A	Riboflavin aldehyde-forming enzyme, F	Molecular chaperone, F

Drying sample groups vs. Dry						
	Up D1h vs. Dry	Down D1h vs. Dry	Up D3h vs. Dry	Down D3h vs. Dry	Up D6h vs. Dry	Down D6h vs. Dry
Annotation	Nuclear pore complex subunit Nup192, putative, F	Ion channel, F	Nuclear pore complex subunit Nup192, putative, F	Ion channel, F	Nuclear pore complex subunit Nup192, putative, F	Ion channel, F
	UDP-glucose 4-epimerase, F	Nitrite reductase, F	T-complex protein 1, gamma subunit, F	Amino acid permease, putative, F	Phospholipase, F	Siroheme synthase, putative, F
	Karyopherin, F	Siroheme synthase, putative, F	Phospholipase, F	Siroheme synthase, putative, F	DDHD domain protein, F	Siroheme synthase, F
	Swr1p complex component, F	Siroheme synthase, F	Swr1p complex component, F	Translation initiation regulator, putative, F	T-complex protein 1, gamma subunit, F	Amino acid permease, putative, F
	Cytochrome P450 family protein, F	Siroheme synthase, N-terminal domain containing protein, F	DDHD domain protein, F	Cation-transporting ATPase, F	Cytochrome P450 family protein, F	Cation-transporting ATPase, F

Drying sample groups vs. Wet						
	Up D1h vs. Wet	Down D1h vs. Wet	Up D3h vs. Wet	Down D3h vs. Wet	Up D6h vs. Wet	Down D6h vs. Wet
Annotation	Dynamin GTPase, A	Nitrite reductase, F	Phospholipase, F	Cation-transporting ATPase, F	Phospholipase, F	Cation-transporting ATPase, F
	Major royal jelly protein, F	Siroheme synthase, N-terminal domain containing protein, F	Mitochondrial carrier protein, putative, F	Translation initiation regulator, putative, F	Mitochondrial carrier protein, putative, F	Hsp98, F
	CaaX farnesyltransferase alpha subunit, F	Hsp98, F	Nitrogen metabolite repression regulator, F	Hsp98, F	Benzoate 4-monooxygenase cytochrome P450, F	Ferric reductase NAD binding domain containing protein, A
	U5 snRNP component, putative, F	Translation initiation factor IF-2, F	U5 snRNP component, putative, F	Ferric reductase NAD binding domain containing protein, A	MFS monosaccharide transporter, putative, F	bZIP transcription factor HacA, F
	Vesicle coat complex COPII, subunit Sec24 family protein, putative, F	Ion channel, F	MFS monosaccharide transporter, putative, F	Polyketide synthase, putative, F	U5 snRNP component, putative, F	Ion channel, F

potentially reflect this activation of pools of transcripts already available in the lichen tissue.

As the rehydration continues, biosynthetic and metabolic processes become more active in *Cladonia rangiferina* as suggested by the enriched GO terms in the W30m sample. These processes are also active in the W1h sample but it seems that these biosynthetic and metabolic processes are less active in the Wet sample than in the W30m and W1h samples. Only GO terms related to nucleic acid binding are enriched throughout the entire rehydration process.

Surprisingly, heat shock protein HSP98 was up-regulated and response to stress GO term enriched in the Wet sample potentially suggesting that three hours' full hydration is sufficient for it to become a source of stress for *C. rangiferina*. Heat shock proteins are induced in various environmental stress responses (Verghese et al., 2012), and it has been shown that the storage of desiccation-tolerant lichen species in moist conditions for several hours is mildly stressful (Minibayeva and Beckett, 2001). Longer periods of full rehydration may potentially be a stressful situation for desiccation-tolerant lichens, as they are generally more adapted to long intervals of water deficiency interspersed with short periods of hydration.

The results suggest that some genes, like nuclear pore complex subunit Nup192, Swr1p complex component and cytochrome P450 family protein, are involved in both the rehydration and dehydration process (Table 4). Nup192 is an evolutionarily conserved nucleoporin, a class of molecules which have been shown to be involved in gene regulation (Capelson et al., 2010). Nucleoporins are necessary for plants to tolerate cold stress (Dong et al., 2006), and essential for the formation of a symbiotic relationship with mycorrhizal fungi (Kanamori et al., 2006). Swr1p allows for a rapid activation of transcription in yeast (Wan et al., 2009), and a quick activation of transcription would seem like an important characteristic in a lichen species, which often experiences short bursts of hydration amid periods of desiccation. Swr1p is a member of the Swi2/SNF2 family (Kobor et al., 2004), and a gene encoding a SNF2 domain-containing protein has been identified as dehydration-upregulated in the resurrection plant *Xerophyta humilis* (Collett et al., 2004). Cytochrome P450 proteins catalyse a plethora of reactions in the primary and secondary metabolism of plants (Mizutani and Sato, 2011, Pinot and Beisson, 2011) and fungi (Ichinose, 2012, Crešnar and Petrič, 2011), and species-specific P450 proteins are often required for the production of secondary metabolites (Mizutani, 2012). A transcript encoding a cytochrome P450 enzyme has been shown to significantly accumulate in moss *Tortula ruralis* during rapid dehydration and subsequent rehydration (Oliver et al., 2009).

The gene expression profiles are similar between the later stages of rehydration and the early stages of dehydration, whereas changes in expression profile can be observed after three to six hours of dehydration although many biosynthetic and metabolic processes are still active in the lichen. Acyl-transferase activities increased during the later stages of dehydration and a U5 snRNP component gene was up-regulated during the entire drying process (Table 4). Small RNAs and ribosome binding proteins are

involved in regulating plant responses to abiotic stresses (Lorković, 2009, Dinakar et al., 2012) and in *Arabidopsis* cold stress induces the expression of a gene encoding a U5 snRNP-associated protein (Lee et al., 2006).

A gene encoding for an ion channel was found to be up-regulated in the Dry sample. Maintaining the integrity of the cell wall and the plasma membrane can be crucial for survival, as the main cause of death during desiccation may be damage to the plasma membrane (Casteriano et al., 2013). This observed increase in the expression level of a transcript coding for an ion channel protein could potentially reflect the protection of cellular integrity that is shown to be integral for desiccation tolerance in plants (Dinakar et al., 2012).

6 CONCLUSIONS

The aim of this thesis was to study the complete gene space of the grey reindeer lichen, *Cladonia rangiferina*, and to identify the molecular mechanisms underpinning lichen desiccation tolerance. Lichens have not been much studied by genomics techniques and while containing large amounts of secondary metabolites the first challenge was to obtain sufficient high-quality material to enable subsequent high-throughput transcriptomics analyses. Several RNA extraction methods were compared and optimised to find the method best suitable for different RNA extraction purposes.

By *de novo* assembling the *Cladonia rangiferina* transcriptome using high-throughput next-generation sequencing data and traditional EST sequences I was able to characterise the functions and pathways active in a wetted lichen tissue through functional annotation with GO and KEGG databases. The assembled sequences were classified into sequences of either algal or fungal origin providing an indication of the ratio between fungal and algal participation in the symbiosis at the transcriptional level. The results give a preliminary view into the molecular nature of the lichen symbiosis and the transcriptional space of this resilient organism.

These transcriptome sequences were further exploited by designing a custom lichen microarray. This was used to study the changes in gene expression during different stages of rehydration and dehydration. The biggest changes in gene expression were detected during the earliest stages of rehydration, and were found to differ clearly from the later stages of rehydration and dehydration. The most differentially expressed genes lacked sequence similarity to functionally characterised proteins or their genes indicating that they are potentially lichen-specific genes, and may contribute to the molecular mechanisms required by the organism to tolerate long periods of desiccation. Several hundreds of genes showing patterns of statistically significant differential expression during either the rehydration or dehydration process were identified. Many of these genes have orthologues or homologues that have been shown to participate in environmental stress tolerance in other organisms.

Finally genomic DNA derived from the *Cladonia rangiferina* thallus was sequenced and the fungal genome was *de novo* assembled and characterised. I was able to obtain an assembly, which is of sufficient quality and includes most of the core eukaryotic conserved genes, and based on the sequence classification and BLAST top hit results, this assembly contains mostly genes derived from the mycobiont. The whole sequence collection certainly contains reads from the algal partner and the bacterial community within the thallus but this information has not been included in the assembly most likely due to the dominating nature of the mycobiont in the lichen symbiosis. The size of the assembled fungal genome was similar to other lichen-forming fungal genome assemblies and several fungal PKS and NRPS genes were present in our assembly and a significant majority of the predicted genes were homologous to known proteins and contained known protein motifs.

While the results of this doctoral work provide a glimpse into the molecular mechanisms behind lichen desiccation tolerance, they are not enough to draw conclusions on the key genes contributing to desiccation tolerance in *Cladonia rangiferina*, and more research is still needed to gain a better understanding of this interesting phenomenon. The decreasing costs of next-generation sequencing offer good opportunities to study even non-model organisms with reasonable funding. However, the bottleneck in non-model organism research is the challenges of *de novo* genome assembly and the lack of reliable functionally characterised protein sequences that genes can be annotated against.

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