Progress in Botany 83

Ulrich Lüttge · Francisco M. Cánovas ·
María-Carmen Risueño ·
Christoph Leuschner ·
Hans Pretzsch *Editors*

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Progress in Botany is devoted to all the colourful aspects of plant biology. The annual volumes consist of invited reviews spanning the fields of molecular genetics, cell biology, physiology, comparative morphology, systematics, ecology, biotechnology and vegetation science, and combine the depth of the frontiers of research with considerable breadth of view. Thus, they establish unique links in a world of increasing specialization. Progress in Botany is engaged in fostering the progression from broad information to advanced knowledge and finally deepened understanding. All chapters are thoroughly peer-reviewed.

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María-Carmen Risueño • Christoph Leuschner •
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Editors

Progress in Botany Vol. 83

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1992	Habilitation in Botanical Systematics, University of Hamburg

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1984–1985	Researcher at Inst. Nat. de Pesquisas da Amazônia (INPA), Manaus, with DFG grant
1985–1987	Postdoctoral Fellow, Smithsonian Institution, Washington, DC, USA
1987–1992	Associate Professor, Botanical Institute, University of Aarhus, Denmark
1993–1996	Professor, Botanical Institute, University of Mainz, Germany
1996–2003	Professor, University of Missouri-St. Louis, MO, USA

2003–October 2020	Professor (chair), Biology, Ludwig-Maximilians University, Munich, Director of the Munich Botanical Garden and of the herbaria in Munich (M and MSB)
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Elected foreign member of the *Royal Danish Academy of Sciences* (2005)
Elected member of the *Bavarian Academy of Sciences* (2009)
Elected member of the *German Academy of Sciences*, Leopoldina (2009)
Elected member of the *American Academy of Arts and Sciences* (2018)

Plant Evolution and Systematics 1982–2022: Changing Questions and Methods as Seen by a Participant

Susanne S. Renner

Contents

Abstract This review describes, in chronological order, the research topics in which I have been involved over the past 40 years, a time during which the study of plant evolution, systematics, and biodiversity has moved from relying solely on morphology to relying mostly on DNA sequences and now partially assembled genomes. When I began to do systematics, traveling to tropical countries for fieldwork was a big draw and probably influenced my initial choice of plant groups to work on. In 1989, I made a conscious decision to shift my focus from monographs, floras, and herbarium-based species discovery to the evolution of plant sexual systems and the functioning of unisexual flowers, selecting first Siparunaceae and then Cucurbitaceae as suitable groups. I also became an early adopter of

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molecular clock approaches in the study of biogeography and plant/animal mutualisms, and was involved in the discovery of natural horizontal gene transfers in seed plants, which in turn led to an interest in mitochondrial and plastid genomes in parasitic plants. Three topics, bee behaviour on flowers, the evolution of ant/plant interactions, and plant phenology, have accompanied me from my dissertation to the present, while others, such as molecular cytogenetics, grew from the interests and expertise of students. The breadth of topics reflects a great change in systematics since the 1980s, namely the increasing role of collaborations. Monographs, floras, and cladistics (when morphology based) used to be done in isolation. With DNA data came lab work, bioinformatics, and both the need and the possibility to collaborate, which brought systematists out of their niche, gave comparative biology a huge push, and resulted in a better integration of biodiversity studies within biology.

Keywords Bees, Biogeography, Botanical gardens, Molecular clocks, Molecular cytogenetics, Natural horizontal gene transfer, Phylogenetics, Plant/animal mutualisms, Sexual systems, Species discovery, Stable naming

1 Introduction: Why I Studied Biology and Ended Up in Systematics

My parents were interested in history and writing, rather than science, but after buying a newly built house surrounded by raw soil in Schleswig-Holstein, my Swabian father developed an interest in gardening. He did this from a book, ‘Der praktische Gartenfreund’ (I still have it), and as a result the garden had all the ingredients fashionable then, such as odd ornamental conifers, a magnolia, *Delphinium,* and ‘Gloria Dei’ roses in front. My father would explain the etymology of the plants’ scientific names, and to this day, I cannot look at a *Hydrangea* without hearing my father’s voice, explaining the name’s meaning. In high school, in Norderstedt, German and biology were my favourite subjects, especially behavioural biology. I read the popular books of Karl von Frisch, Konrad Lorenz, Nikolaas Tinbergen, and Irenäus Eibl-Eibesfeldt, and a mini-research project, when I was 15 or 16, on ‘Sex symbols in advertisement’ in hindsight set me on my path (Sect. 7).

In the summer of 1972, my parents let me spent 6 weeks in Gananoque, Ontario, where I helped an uncle who was a forester, beekeeper, and budgie breeder. The next summer, when I had finished high school, they let me visit another uncle, who worked in Santiago de Chile. After a couple months of volunteering, I began studying marine biology at the University of Chile. This came to an end when the university closed because of the murder of Allende in September 1973, and I will never forget how in early October, my uncle’s house was searched by two young men probably my age (19), toting machine guns. After that, I decided to continue my studies at the university of Hamburg. The most wonderful classes for me were those

Fig. 1 Klaus Kubitzki instructing Susanne Renner during her M.Sc. research in 1979 (private photo)

of the zoologists, including Wolfgang Villwock (1930–2014) on fishes, Hans Strümpel (1935–2019) on social insects, and Otto Kraus (1930–2017) on spiders. Kraus, of course, introduced us to Willi Hennig and cladistics.

Unfortunately, the only professor in animal behaviour, which I had planned to specialize in, let us do experiments on imprinting that should never have been permitted. One involved three students, including myself, sleeping next to an incubator with chicken eggs so that we might imprint the newly hatched chickens on a ball. The chickens, sadly, never walked at all because they all had splayed feet and crippled legs. Nobody had told us that just-hatched chickens must never be kept in containers with smooth surfaces. This experience drove me to botany.

Botany in Hamburg was well-represented by, among others, Konrad von Weihe (1923–2013), who taught economic botany (in which I then wanted to specialize), Hans-Dieter Ihlenfeldt (1932-), Heidrun Hartmann (1942–2016), and Klaus Kubitzki (1933-), who taught systematics and evolution. The seminar classes offered by professor Kubitzki, especially one on flower/pollinator coevolution, and an excursion he led to Obergurgl in the Austrian alps stood out. Kubitzki knew every plant and every moss in Obergurgl, but realizing that I knew nothing, he happily demonstrated *Poa annua* to me, explaining that students from Hamburg actually best learn this species on Mount Etna.

Klaus Kubitzki and I shared a love for South America, especially Chile and Brazil. I had spent the summer of 1978 in São Paulo and Rio de Janeiro, with a great-aunt, and so by then I could get by in both Spanish and Portuguese. After my oral examinations in entomology and botany, I therefore embarked on an M.Sc. thesis (1978/79; Fig. 1) focused on *Aiouea*, a South American genus of Lauraceae, under Kubitzki's guidance, later published in the *Flora Neotropica* series of the New York Botanical Garden (Renner 1982). Disappointingly, the project involved Kubitzki traveling in South America and me going through the hundreds of specimens he had on loan in the herbarium in Hamburg. To identify Lauraceae, one has to boil their minute flowers in hot water and then dissect and draw them under a stereoscope fitted with a drawing tube. I was not keen on this. The best part for me instead consisted in extracting information from the labels. Using label data, I found that two morphologically similar and co-occurring species differed in their flowering time. I also placed every collection on the standard *Flora Neotropica* maps, which required studying old maps and finding out about the collectors' itineraries. While none of this was earth-shattering, my approach taught me how to use label data to study phenological niches, starting an interest that would lead to some of my most innovative research.

For my doctoral research, begun in 1980, professor Kubitzki let me chose between ant/plant interactions or bee/flower interactions. I decided on the latter topic, realizing that Melastomataceae would afford me an opportunity to also study the former (ant plants came back into focus later; Sect. 5). I moved to Manaus for 25 months, doing fieldwork on the reproductive biology of Melastomataceae, which involved traveling all over the Amazon basin and also to Minas Gerais. Part of my work was done within the Minimal Critical Size project north of Manaus, run by Thomas Lovejoy (1941–2021), a leading thinker in the study of 'biological diversity', a term that he coined and which his friend E. O. Wilson (1929–2021) then shortened for a 'National Forum on BioDiversity' at the Smithsonian Institution in Washington in September 1986 that I was fortunate to attend as a postdoc. The Minimal Critical Size project brought numerous visiting researchers from the USA, working on army ants, snakes, frogs, birds, and other animals variously able, or unable, to traverse the burnt strips of land between the blocks of left-over forest (the 'minimal' reserves). It also mapped and tagged thousands of trees, which meant I could do ecological observations, including monitor flowering and fruiting times, another topic to which I returned 20 years later, when changing phenology under climate change became a concern (Sect. 8).

The external examiner for my dissertation, written in German and on a typewriter, and oral defence, back in Hamburg in late 1983, was Stefan Vogel (1925–2015). To prepare, I read all his publications, and, I think, Stefan Vogel is among the biologists who had the deepest influence on me. We stayed in close contact until late 2012. The other person to whom I owe the greatest debt is Klaus Kubitzki, who 4 years after my Ph.D., when I had my first academic position at the University of Aarhus in Denmark, supported my habilitation at the University of Hamburg, which I did as a 'traveling professor' from Aarhus.

2 Monographs, Floras, and Herbarium-Based Species Discovery: The Years in Washington, Aarhus, and Mainz

After defending my dissertation, I obtained my first grant from the German research foundation (DFG) to work on the systematics of the tree genus *Bellucia* (Melastomataceae), which took me back to Manaus. From there, I was invited to participate in an American-led expedition to the Serra da Neblina (1984), a tabletop mountain at the border between Brazil and Venezuela. This was a helicopter-dependent adventure, and because of the fickleness of helicopters, we ended up spending 20 straight days between 1,850 and 2,100 m elevation, which gave me time for pollination observations that remain among the few such studies from any tabletop mountain (Renner 1989). I continued my taxonomic work with a postdoctoral fellowship from the Smithsonian Institution in Washington (June 1985–December 1987) where I worked with John J. Wurdack (1921–1998; Fig. 2), a proponent of the importance of collecting and studying species' geographic distribution. Through John, a gregarious person who often invited visitors to lunch at the Smithsonian's 'Castle', I met some of the heroes of the era, including Joseph Ewan (1909–1999), Armen Takhtajan (1910–2009), and Arthur Cronquist (1919–1992).

Fig. 2 My postdoctoral advisor, John Wurdack, on Serra da Neblina, Venezuela, in 1959 with one of the *Heliamphora* plants whose pollination biology I later studied on the same mountain

In 1988, Art Cronquist even took me along on a short plant collecting trip in California during which we camped out under the stars.

Scientifically, I was kind of stuck, monographing ever more genera (*Bellucia*, *Loreya*, *Macairea, Rhynchanthera, Pterogastra*, *Schwackaea, Pterolepis* and more) and contributing treatments to floras and checklists (for Ecuador, Venezuela, Colombia, Thailand, China). Herbarium-focused research continued during my years as assistant, then associate, professor in the botany department of the University of Aarhus (March 1987–December 1992), which had long-established ties with institutions in Greenland, Ecuador, and Thailand, and colleagues there took me along on many field trips, including to Thailand with Kai Larsen (1926–2012), Greenland with Simon Laegaard, and Ecuador with Henrik Balslev.

Thinking about a new direction for my research, I decided to work on plants with sexually specialized sporophytes (dioecious plants) – a sexual system superficially resembling that of animals but making little sense in sedentary organisms, such as land plants. Since I was familiar with Laurales, I chose Monimiaceae, which often have unisexual flowers and which in some classifications included Atherospermataceae, Monimiaceae, Siparunaceae, and even *Amborella* and *Trimenia* (the latter soon excluded and recognized as among the oldest lineages of flowering plants; Renner 1999; Mathews and Donoghue 1999). In Ecuador, one could study the reproductive biology of Monimiaceae and Siparunaceae, and with my first M.Sc. student, Jan Peter Feil (M.Sc. thesis 1989–1990), we did exactly that.

Having published lengthy taxonomic works on various plant groups and with over 100 new species associated with my name, I came to realize that for me, herbarium-based work was too static, even when paired with collecting trips to tropical countries. There are 1.2 million published names for land plants, 46% of which are considered synonyms, and in the flowering plants, there may be 3–4 synonyms for every accepted name. This redundancy, which is a huge problem for evolutionary studies, can only be solved by people trained in finding type specimens, which often involves historic-detective work, and who know how to assess traits, geography, and ideally also DNA data. All my doctoral students, I hope, acquired a basic understanding of this, but only four of 22 (at universities in Aarhus, Mainz, Saint Louis, and Munich) decided to include extensive taxonomic work in their theses: Gudrun Kadereit, née Clausing, who worked on several genera of Asian Melastomataceae and who since the beginning of 2021 holds the Chair of Systematic Botany at the University of Munich (thus being my successor); Karsten Meyer, who worked on the genus *Melastoma*; Norbert Holstein, who worked on the genus *Coccinia* (Cucurbitaceae) and is now a curator at the Natural History Museum in London; and Fernanda Carvalho, who revised the papaya family (Caricaceae) and is now a professor at the university of Minas Gerais in Belo Horizonte. All also generated molecular phylogenies and carried out fieldwork. My own most fulfilling taxonomic work, on the above-mentioned Monimiaceae and Siparunaceae, was done in collaboration with the unforgettable embryologist, mycologist, and born taxonomist Gerlinde Hausner (1939–2008) who worked with me during my 3 years at the university of Mainz (e.g. Renner and Hausner 1997, 2005 another volume in the *Flora Neotropica* series).

3 The Move to Molecular Data: Likelihood, Molecular Clocks, and Biogeography

From mid-1996 onwards, when I moved to the University of Missouri-Saint Louis (UMSL), I focused on obtaining DNA sequences to understand relationships in the 'Monimiaceae' (e.g., Renner et al. 1997, 2000), and my lab therefore produced the first sequences of *Trimenia* and *Amborella*, which fell far outside Laurales (Renner 1999; Mathews and Donoghue 1999). I therefore gave aliquots of the DNAs to Yin-Long Qiu at the University of Michigan and Michael Zanis, then a Ph.D. student in the lab of Pamela and Douglas Soltis, who were working on a large phylogeny of the flowering plants (Qiu et al. 1999, 2005), a sharing that led to visits with top people in the developing field of angiosperm phylogenetics.

With DNA sequences in hand, I became interested in molecular-clock dating and set about learning and testing how to apply this tool, inspired by the work of Sanderson who first used multiple fossil calibrations (Sanderson 1997 and onwards). I believe my paper on the history of the Southern Hemisphere family Atherospermataceae, which has fossils in New Zealand and Patagonia, was the first application of molecular-clock-dating in plant biogeography (Renner et al. 2000; Fig. 3). The basic idea with all molecular clocks is to find a genome region that accumulates substitutions suitably fast (with 'suitably fast' depending on the questions one is asking) so that they can be 'seen' and counted in a DNA alignment,

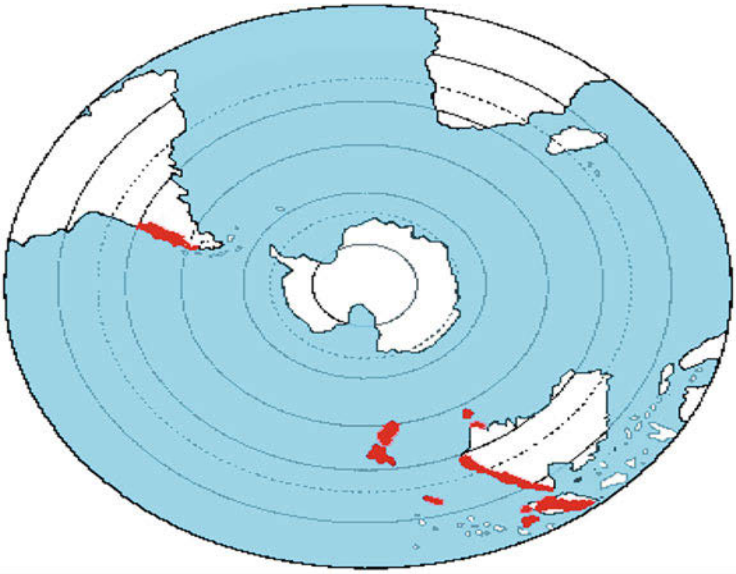

Fig. 3 The distribution of Atherospermataceae, which have two species in Chile and 12 in Australasia, with fossils in Patagonia and New Zealand that we used to calibrate an *rbcL*-based molecular clock (Renner et al. 2000)

and translated into proportional branch lengths (i.e., genetic distances) in a phylogeny. Branch lengths are then calibrated with some external event, such as a volcanic eruption, a characteristic fossil, or the date when a patient's blood was sampled. The obtained rate (number of substitutions per time) can be used to translate the age of any node in the phylogeny into absolute time (in millions of year or years, as the case may be). Modern epidemiology and biology are unthinkable without molecular clocks. Of course, any inferred time has an error range, which can also be calculated. Molecular clocks are the basis for quantifying evolutionary change in absolute time, be it in the SARS-CoV-2 virus human evolution, plant/animal interactions, biogeography, or the first appearance of particular traits of interest, such as carbon-concentrating mechanisms in hornworts (Villarreal and Renner 2012).

Having understood the power of the new approach, my students and I applied clock dating to numerous biogeographic and evolutionary questions, both in case studies (Renner and Zhang 2004 – with a wonderful postdoc, Libing Zhang, now a senior curator at the Missouri Botanical Garden; Won and Renner 2006; Sebastian et al. 2010; Renner et al. 2020) and more general work. The latter included an early review of the so-called relaxed molecular clock approaches (Renner 2005), how to infer the root of a phylogeny, i.e., its evolutionary starting point, with a clock model (Renner et al. 2008), the introduction of fossil-based 'ghost' lineages that prove a former geographic presence no longer seen in any of the sequenced living taxa (Mao et al. 2012; Nauheimer et al. 2012), and the application of the 'Fossilized Birth/Death' model in which all fossils of a clade (not just the oldest ones) are used for calibration (Grimm et al. 2015; Renner et al. 2016).

In 2002, when molecular-clock approaches were beginning to reveal the frequency of successful long-distance dispersal, casting doubt on earlier plate-tectonic-type explanations for geographic disjunctions, Tom Givnish and I organized a symposium at the University of Madison in 2002 on '*Tropical intercontinental disjunctions: Gondwana break-up, immigration from the boreotropics, and trans-oceanic dispersal'* (Givnish and Renner 2004). My own contribution dealt with plant dispersal across the tropical Atlantic by wind and sea currents (Renner 2004), and I argued that despite the influence of chance, meta-analyses should be able to detect predominant patterns in suitable time spans because of the predictable direction of sea and wind currents. Another meta-analysis focused on the 4 km-high Tibetan Plateau, which geologists tend to agree was already that high by 40 Ma ago. Yet, at least 100 studies of plants and animals have claimed that the rise of the Tibetan Plateau occurred a mere few million years ago, matching their various clock-dated young groups of organisms (Renner 2016a; the 100 papers are listed in my paper's appendix). This is entirely due to copy-and-paste science and is in no way a sign that molecular-clock-dating does not work.

4 The Discovery of Natural Horizontal Gene Transfer in Seed Plants; Mitochondria, Plastomes, Transposons, and Finally Nuclear Data for Studying Plant Domestication

Hyosig Won, my second doctoral student in Saint Louis, arrived in my lab in September 1998, directly from South Korea, and it soon became clear that he had a much more solid understanding of molecular biology than I did. He taught me about intron regions, mobile elements, and splicing, and working with him was great. One day, he came and said he had discovered sequences in the mitochondrial DNA of *Gnetum*, the gymnosperm genus on which he was doing his dissertation, that could only come from some plant in the sunflower family. Of course, Hyosig checked everything multiple times before we submitted our paper to *Nature* on 2 February 2003. As expected, the reviewers (on March 5) said that horizontal gene transfer (HGT) among higher plants could not exist. A couple days after this rejection, Jeffrey Palmer from Indiana University called me out of the blue to tell me that he believed in our results and that I should resubmit our paper to the *Proceeding of the National Society*, which we did on 12 June and where it was accepted on 18 July. By coincidence, a paper from Jeff Palmer's lab with the first case of HGT in flowering plants came out in *Nature* on 10 July 2003 (Bergthorsson et al. 2003). All these cases involved standard mitochondrial genes, encoding ribosomal and respiratory proteins, and since 2003, it has become clear that HGT between distantly related land plants has occurred relatively often. The precise mechanisms are still an open question, but most instances involve parasitic plants that are attached to their host via plasmodesmata through which phloem sap, macromolecules, and entire mitochondria can pass.

For several years, HGTs of mitochondrial genes among higher plants was an exciting area, and when I moved to the university of Munich in June 2003, people in my lab kept an eye open for additional cases. One of my first doctoral students in Munich, Natalie Cusimano, who came from Berlin in February 2005, studied the acquisition and loss of horizontally-transferred mitochondrial introns in Araceae (Cusimano et al. 2008) and later, after we began using Next-Generation sequencing (NGS) in 2011, Natalie and I investigated HGT events between the endoparasite *Cynomorium coccineum* and its various hosts (Cusimano and Renner 2019; Fig. 4). Another student to focus on parasitic plants was Sidonie Bellot, who joined in 2010, coming from an M.Sc. project in France where she had already used NGS and knew how to analyse the data. Sidonie focused on the plastid genomes of the worldwide endoparasite family Apodanthaceae, which led to the discovery that these parasites have lost all but five or six of the 110–130 genes in a typical angiosperm plastome (Bellot and Renner 2016). This may still be the smallest plastid genome known in the embryophytes.

Better sequencing machines eventually allowed systematists to routinely obtain nuclear sequences, but much of what one obtains from next-generation sequencing (NGS) consists of transposons and other types of repetitive DNA. But how to use

Linnaeus, Species plantarum 1753

CYNOMORIUM.

coccineum. 1. CYNOMORIUM. *Mat. med.* 534. 1749
Cynomorium purpureum officinarum. *Mich. gen.* 17. *t.* 12. 1729
Fungus mauritanicus verrucoſus ruber. *Petiv. gaz. t.* 39. *f.* 8 ca. 1702
Fungus typhoides coccineus tuberoſus melitenſis. *Bocc. muſ.* 2. *p.* 69. *fic.* 80. *t.* 81. 1697
Fungus typhoides liburnenſis. *Till. piſ.* 64. *t.* 25. 1723
Habitat in Sicilia, Melita, Mauritania *paraſiticum terreſtre.*

Fig. 4 The inflorescence of a *Cynomorium coccineum* plant, a root holoparasite that Linnaeus and his contemporaries thought was a fungus, as is evident from the synonymous names given in Linnaeus's 1753 *Species Plantarum*. The species occurs in sandy soils from Spain to China and parasitizes a limited number of hosts, which enabled us to study its sequential acquisition of mitochondrial genes from different hosts over geographic space and geologic time (Cusimano and Renner 2019). The species is in its own family, which we placed in the Saxifragales (Bellot et al. 2016)

these difficult-to-align data in comparative biology? One option was to focus on transposons and the question of how they are inherited. Some transposons are highly labile, even during the lifetime of an organism, others are not. With a French postdoc, Mathieu Piednoel, with deep experience in retrotransposons, I submitted a grant request on this topic to the DFG, and when we were funded, Mathieu joined my lab for 3 years (2011–2014). Because of my interest in parasitic plants, we focused on Orobanchaceae, sending material of selected autotrophic, hemi-, and holo-parasitic diploid and tetraploid species to a company for NGS as well as generating a 'traditional' phylogeny in our own lab from short sequences and with dense species sampling. We discovered that major plant LTR-retrotransposon families undergo taxon-specific proliferation are especially chromoviruses of which we found species-specific families (Piednoel et al. 2012, 2013).

The Cucurbitaceae, a family of about 1,000 species with an exceptional number of economically-important crops, wide geographic occurrence, tight coevolutionary interactions with various groups of bees (Sect. 6), and unisexual flowers in monoecious or dioecious species (Sect. 7), became a focus from June 2001 onward. However, obtaining good material took years because many cucurbits are tropical climbers or desert-adapted plants that are difficult to collect because they only emerge from their underground tubers or leaf-less thick stems with sufficient rain. Once it became easier to obtain nuclear sequences from herbarium material, my students, postdocs, and I started making many unexpected discoveries about the wild relatives (and their geographic distribution) of cucumber, watermelon, honey melon, and others (Renner et al. 2007a, b; Sebastian et al. 2010; Chomicki and Renner 2015, Chomicki et al. 2020d: a review of Cucurbitaceae domestication). Genomic data are crucial for resolving the evolution of cultivated species because the events are so young, usually having occurred over the past 10,000 years. Inferring the domestication of the watermelon therefore took a multi-year, multi-postdoc, multi-national project, as well as the classic taxonomist's detective skills to check old maps, labels, type locations, and small, but important herbaria. It turned out that Sudanese wild melon are the most likely wild progenitors of the domesticated watermelon (Renner et al. 2021b).

I am very grateful to students and collaborators who enabled me to step into the phylogenomic era, foremost among them Guillaume Chomicki (Sect. 5), Oscar Pérez-Escobar, formerly a doctoral student in Munich and now a curator at the Royal Botanic Gardens in Kew, and Wei-Ning Bai and Da-Yong Zhang at Beijing Normal University. With the last two, I am working on the evolution of Juglandaceae, including the walnut, which it turns out originated as a hybrid species, as inferred with population-genetic methods (Zhang et al. 2019).

The functioning of my lab in Munich depended on Dr. Martina Silber, who ran it from January 2010 until my retirement. With her help, we were able to do barcoding, genomics, transcriptomics, and molecular cytogenetics (Sect. 7). She supervised bachelor and M.Sc. students in the lab, was on top of safety protocols, lab inspections, and the orderliness of purchases and budgets, and was genuinely interested in the different questions we were working on, exploring new methods on her own, with much more technical know-how than any of us others. Without Martina, the lab's success would not have been possible.

5 Functional Morphology, Plant/Animal Interactions, and Discovery of Ant-Cultivated Rubiaceae

The evolution of flowering plants cannot be understood without considering their interactions with pollinators because there lies the key to the origin and function of flowers. Early pollinators, back in the late Jurassic and early Cretaceous when flowering plants evolved, must have been certain flies, beetles, and moths that searched out gymnosperm cones as mating sites and for egg-laying. These insects' visits to male and female cones of now extinct gymnosperms must have contributed to efficient (because directed) pollen transfer, setting up the context for the mutual adaptions and coevolutionary divergences that led to the diversity we see today. These topics have always played a large role in my teaching (in Aarhus, Saint Louis, and Munich), and over the years, M.Sc. students in my lab and students whom I co-advised have carried out studies on pollination by flies and beetles in *Nuphar* in Scandinavia, Germany, and North America, by midges in Ecuador and China, and by pollen-feeding moths in China. This work – along with studies from others – showed that early-evolving angiosperm groups that still survive are mostly pollinated by ovipositing flies. Compared to food reward-based pollination, oviposition-based systems are less wasteful of plant gametes because female insects with herbivorous larvae reliably visit the particular oviposition sites (i.e. flowers) to which their larvae are adapted (Luo et al. 2018).

Collaborations in the field of plant/pollinator interactions came from working with Chinese students and postdocs who over the years became close colleagues, especially Dianxiang Zhang and Shi-Xiao Luo of the botanical garden in Guangzhou and Shuang-Quan Huang from Central China Normal University in Wuhan. The projects involved mutual visits and field trips, seminars at my Chinese host institutions, and lots of wonderful Chinese food. My very first visit to China, in April 2004, yielded the discovery of a new self-pollination mechanism by pollen grains that are sliding down the flower's own style and onto its stigma (Wang et al. 2004). Another project that stands out in my mind involved experiments on the function of morphologically differentiated stamens in buzz-pollinated flowers (Luo et al. 2008). The results supported Darwin's division-of-labour hypothesis, which predicts that the pollen grains from the set of anthers less conspicuous to bees should have a higher chance of reaching a conspecific stigma than the grains from the other more conspicuous set.

Functional analyses often require microscopy and stereoscopy, something of which I felt I had done quite enough during my M.Sc. on the genus *Aiouea*, which has tiny nectar glands like most Lauraceae (Sect. 1). However, a collaboration with the morphologist Florian Jabbour, who spent 2 years in my lab (2010–2012) as a postdoc with funding from the DFG rekindled my interest in the functioning of nectaries, this time inside the complex perianth of the Delphinieae (Ranunculaceae), a group that Florian is an expert on (Jabbour and Renner 2012a, b). Florian is now working at the Museum National d'Histoire Naturelle in Paris, with a continued focus on the Ranunculaceae. Because of their huge role in plant/pollinator interactions, nectar spurs and nectar production remained a focus in my work. With an M. Sc. student, I applied molecular-clock dating to the African moths and the long-spurred *Angraecum* star orchids that fascinated Darwin, and we discovered that – against my expectation – the longest-spurred orchids and the longest-tongued *Xanthopan* moths on Madagascar are of the same age, namely ~7 my, supporting that their extreme traits are the result of coevolution, despite the orchid probably being more dependent on the moths than the other way around (Netz and Renner 2017; more field work is needed).

Nectar selectively offered in suitable spurs also is the sine qua non in hummingbird pollination, a topic on which I worked with another postdoc, Stefan Abrahamczyk (2012–2014), now at the Natural History Museum in Stuttgart. We investigated the timeframe over which hummingbirds, which provide great pollination services and thereby 'drive' flower evolution, have interacted with different

Fig. 5 The sword-billed hummingbird, *Ensifera ensifera*, with an up to 11 cm long bill, visiting *Passiflora mixta.* With molecular clocks applied to a hummingbird phylogeny and several plant phylogenies we inferred that this hummingbird diverged from its sister species ~11.6 million years ago and that at least 45 plant species in seven genera and five families now depend on this bird for pollination, many of them having evolved in a step-wise process. Photo Rolf Nussbaumer, Ecuador

plant genera and even entire families, leading to classic cases of convergent evolution, the parallel acquisition of similar traits in unrelated species. The results show that this can take just a few million years, followed in some groups (such as the Passifloraceae that we focused on) by 'evolutionary escapes' from ancestral bird pollination to pollination by bats, moths, or bees (Abrahamczyk et al. 2014; Abrahamczyk and Renner 2015; Fig. 5).

In April 2013, Guillaume Chomicki visited to discuss possible topics for a doctoral project. Within 30 min, we discovered a shared interest in ant/plant interactions, which had interested me since my own doctoral research (which included observations of the ant-occupied genus *Tococa*) and ever since (Renner and Ricklefs 1998). We both thought that the evolution of ant/plant symbioses, the permanent physical living of ants inside plant-formed nesting structures, had not been studied with the full arsenal of comparative biology, including field experiments, lab-based developmental studies, molecular-phylogenetics (including transcriptomics, Pu et al. 2021), and molecular-clock dating. As the focal ant/plant clade, we selected a group of Rubiaceae with about 100 species in SE Asia, with fieldwork to be carried out in the Fiji Archipelago (funded by our DFG grants). Guillaume did not shy away from rope-climbing trees, then hanging in a harness to reach his epiphytic study plants with their ant-housing tubers, which of course were aggressively defended by the ants. He was as ingenious in the lab as in the field, for example, applying computer-tomography to study the inside of the ant-housing tubers, and just a fantastic student.

Guillaume's work led to the discovery of obligate insect agriculture, involving the symbiosis between the ant *Philidris nagasau* and epiphytes in the genus

Squamellaria (Rubiaceae), which the ants plant, fertilize, defend, harvest, and depend on for nesting. Before our project, farming systems (outside human agriculture) had only been known to involve fungi cultivated by social insects, but never plants obligately depending on ants throughout their life cycle (Chomicki and Renner 2016, 2017a, b). The relationships between *P. nagasau* and several *Squamellaria* are reciprocally obligate and evolved about 5 million years ago, as we inferred from clock-dating a phylogeny for the ants and another for the plants. Guillaume and I have explored many aspects of the ants' and plants' mutual adaptations, such as the flowers' concealed nectar, exclusively available to *P. nagasau* (Chomicki et al. 2016) or the different trade-offs in the symbiosis in sun or shade environments (Chomicki et al. 2020a), and we are continuing to collaborate. Now in his own lab at the University of Sheffield, Guillaume is asking more general questions about the evolution of mutualistic dependence and its role in diversification (e.g., Chomicki et al. 2020b, c).

6 Bee Phylogeny and Behaviour, and a Botanical Garden as a Terrarium

I had originally wanted to study animal behaviour, especially in bees, probably because of Karl von Frisch's work on bee communication (von Frisch 1965; Sect. 1), and my first-ever paper was on pollen-stealing bees-stealing because the thieves do not pollinate the flowers whose pollen they steal (Renner 1983; Fig. 6). This topic – deception in mutualisms, with one partner gaining a benefit without 'paying' the other – is fascinating, but requires mathematical modelling for a deeper understanding, and for lacking this skill, my forays into deception and ecological (phenological) mismatch have mostly remained meta-analyses or reviews (Renner 2006; Renner and Zohner 2018; Chomicki et al. 2020b).

An opportunity to again work on bees came when Hanno Schaefer visited to discuss possible postdoctoral projects in late 2004. Hanno, now a professor at the Technical University in Munich, has an incredible knowledge of the diversity and ecology of temperate and tropical bees, birds, and plants, and since we both were fans of the work of Stefan Vogel (Sect. 1) on oil bees and the oil-offering flowering flowers, we settled on this topic and got a DFG grant. Among the results of this work was a phylogeny of the Ctenoplectrini bees, a clade of 20 species in Africa, Asia, and Australia that has coevolved in space and time with oil-offering Cucurbitaceae (Schaefer and Renner 2008) and a study of how the various mutualistic systems of oil-collecting bees and oil-offering flowers in South America, Africa, Asia, and Europe originated and diversified, using other plant/bee interactions as stepping stones (Renner and Schaefer 2010). Many of our insights were due to Hanno's field work, my molecular-clock expertise, and the ground-breaking work of Stefan Vogel (Sect. 1).

Fig. 6 Examples of the pollen-robbing behaviour discovered during the fieldwork for my dissertation (Renner 1983). A. *Trigona williana* cutting into an anther of *Rhynchanthera grandiflora*. B. A bud of *R. grandiflora* damaged by *Trigona* bees trying to get at its pollen before the flower even opened. C. *Tococa longisepala* flower with 9 of its 10 anthers completely cut off by *Trigona* bees. D. *T. fulviventris* destroying the anthers of *Tibouchina maximiliana* to get at the pollen grains. All Photos: S. S. Renner

Further work on oil bees, this time from South America, was possible when Aline Martins, a sandwich Ph.D. student from Brazil joined the lab for a year (2013/2014). We investigated the coevolution and geographic range overlap between various oil bee subgroups and South American plant families (Martins et al. 2014, 2015).

My last doctoral student, Michaela Hofmann (2017–2020), also worked on bees, this time, bees in Germany, with a focus on niche occupation, food competition between wild bees and honeybees, and experiments in the Munich botanical garden. One of our discoveries was that in Germany (with 445 species for which we had data), bee species that forage in the spring are less extinction prone than species that forage later in the year and that bees capable of living in urban environments (82 species) are less extinction prone than the 346 species specialized to live in various non-urban habitats (Hofmann et al. 2019). We were able to quantify local extinction because of Germany's 'Red List' assessments, which have been carried out regularly for the past 40 years, usually by the same few experts (for different taxa).

With Andreas Fleischmann, my colleague in Munich and another bee expert, we took advantage of the Munich botanical garden being a kind of terrarium, that is, a system whose components are known and change in pretty well-understood ways, for example, due to climate warming. The garden, which covers 21 ha and opened in 1914, has not changed its basic lay-out and for the past 100 years and is home to 110 species of bees that are well monitored. This permitted us to attribute the strong increase in warm-habitat loving bee species since 1997 to climate warming rather than increase in food or nesting sites (Hofmann et al. 2018). Michaela and Andreas also taught several cohorts of bachelor students how to label tiny bees with numbers or colour codes, so we could study foraging distances. Foraging distances are well studied for bumble bees (and of course the honey bee), but 92% of the 561 species that occur in Germany are only 4.5–13.5 mm long, and by studying the foraging distances in six species with body lengths of 6–15 mm, we developed a 150-m-rule-of-thumb for flower strip distances (Hofmann et al. 2020). With another two cohorts of bachelor students, Andreas and I also studied food competition between honey bees and wild bees, discovering a clear displacement effect in a year when honey bees were more abundant than in a previous year (Renner et al. 2021a, b).

7 Plant Sexual Systems and Molecular Cytogenetics

The evolution and function of unisexual flowers has been one of my main topics (Sect. 2), with a focus on dioecy and monoecy, the distribution of unisexual flowers on separate individuals or on each individual (in populations). My first forays into the field were reviews of the distribution of dioecy and monoecy in the flowering plants, pollinators of tropical dioecious angiosperms – most dioecy is found in long-lived tropical plants – and the occurrence of heteromorphic sex chromosomes in land plants (Renner and Feil 1993; Renner and Ricklefs 1995; Ming et al. 2011; Renner 2014). A step forward came with the doctoral research of Stefanie Volz, who studied monoecy, dioecy, and chromosomes in *Bryonia*, the Cucurbitaceae genus in which Carl Correns in 1903 discovered the Mendelian inheritance of sex by counting the male and female offspring from crossings that he carried out between monoecious and dioecious species. Among my many adventurous students, Stefanie stands out by traveling to the deserts of Uzbekistan to dig for *Bryonia* tubers so as to bring them into cultivation in the Munich botanical garden for chromosome counts.

An insight emerging from our phylogenetic studies was how often monoecy and dioecy have gone back and forth during evolution. This finding gradually became a leitmotif in my understanding of sexual system evolution: The biggest hurdle for flowering plants is to achieve reliable pollination with unisexual flowers, after which switches between monoecy and dioecy are evolutionarily easy (Renner and Won 2001; Renner et al. 2007a, b; Volz and Renner 2008; Schaefer and Renner 2010; Renner 2016b; Renner and Müller 2021). My persistent stressing of the 'monoecy pathway' to dioecy, which arose from the taxa I was working on (Laurales and Cucurbitaceae), became a much-cited contribution to the field.

The advent of NGS resulted in the easy amplification of repetitive DNA (Sect. 4), which when labelled with fluorescent molecules and hybridized onto chromosomes

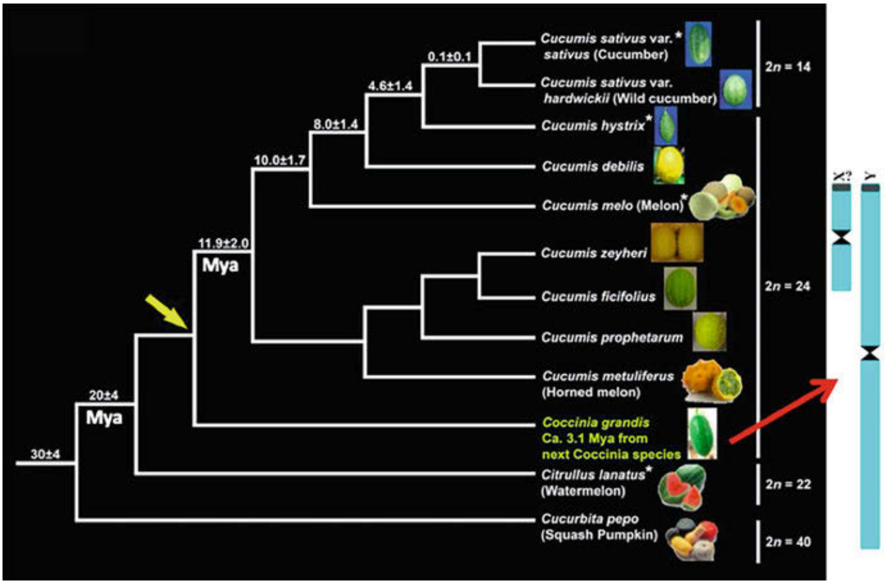

Fig. 7 A partial phylogeny for the Cucurbitaceae showing some of the family's many crop species and the placement of the genus Coccinia, all species of which are strictly dioecious, with at least two of them having an extremely large Y chromosome (image created by A. Sousa in 2013)

can serve to distinguish individual chromosomes (under fluorescent light). This molecular-cytogenetic technique, called FISH, was developed in the 1980s, but in plants applied only from 1989 onwards because plant cell walls make it hard to obtain good chromosome preparations, which are the sine qua non for successfully 'painting' chromosomes. Chromosome researchers in Poland, Vienna, and the former East Germany were leaders in this field, and they trained students in molecular cytogenetics. One of them started his own cytogenetic lab in Brazil, and by sheer luck, a M.Sc. student from his lab, Aretuza Sousa, applied to work with me. She opened up an entire field not only for me, but also for several graduate students and one of my colleagues, who all enlisted her help for cytogenetic studies on their groups of interest.

With Aretuza's molecular-cytogenetic know-how, we studied fusion of chromosomes during evolution (inferred when telomeric fluorescent markers suddenly appear in the middle of chromosomes), changes in chromosome numbers during evolution using a new maximum-likelihood model (Cusimano et al. 2012), the UUV sex chromosomes of liverworts (Renner et al. 2017; Sousa et al. 2021), and the sex chromosomes of species of *Coccinia* (Sousa et al. 2016, 2017; Fig. 7). This last topic continues to fill my days, with ongoing collaborative work on the evolution of the huge Y chromosome of *C. grandis* by now based on a chromosome-level assembled and annotated genome of this species. The annotation of the function of many of the genes was possible because the cucurbit family contains so many vegetable and fruit crops (Fig. 7) with much research on fruit yield, appearance, and taste.

8 Phenology and Botanical Gardens as Common Gardens

The species-specific flowering times of trees growing near the equator, which in some species are precisely synchronized among thousands of individuals across large geographic regions, have long been noted by tropical botanists. However, the environmental signals used by these plants have not yet been figured out. In my doctoral research, I had documented such synchronized annually repeated flowering in species of *Miconia*, using my own observations and herbarium material to acquire data for earlier years. This brought me in contact with the plant physiologist Rolf Borchert (1933–2017) from the University of Kansas in Lawrence, and we worked together from 1995 until 2009, with mutual visits in Mainz, Saint Louis, Lawrence, and Munich during which we discussed possible experiments to try and figure out the environmental cues. However, even though our collaboration resulted in a paper in *Nature* (Borchert et al. 2005), it ended in frustration because I remained unconvinced that our proposed explanation was completely right. The controversial discussions with Rolf about whether air temperature, precipitation, or the annual day length cycle was the external signal against which tropical plants are calibrating their internal clocks, however, stuck in my mind.

In the spring of 2012, Constantin Zohner came to my office asking about a possible topic for his M.Sc. thesis (in Germany still the obligatory precondition for starting on any doctoral research). I suggested that he should study leaf-out times in the Munich Botanical Garden, observing as many trees and shrubs as possible and relating their leaf-out to the region where they came from. Constantin is an exceptional experimentalist and, equally important, interested in statistics and analytical methods; he is now at the ETH in Zurich, with his own state-of-the-art climate chambers. In Munich, Constantin ended up studying almost 500 woody species (permanently outdoors) from numerous genera, families, with 85% of them not native in Central Europe. The results revealed, for the first time, the permanent footprint that adaptation to local climate leaves on the phenology of tree species (Zohner and Renner 2014). We titled our paper 'common garden comparison of the leaf-out phenology of woody species from different native climates, combined with herbarium records, forecasts long-term change' because we realized that for long-lived species, botanical gardens are equivalent to common garden experiments in giving us the power to separate genotype and phenotype, an experimental approach first developed in the 1930s by one of the fathers of the study of plant adaptation, Göte Turesson, working on bud burst and autumn leaf senescence in Sweden, and Clausen et al. (1940), working in California. Amazingly, nobody had ever applied the concept to the arboreta of botanical gardens.

The insight that botanical gardens, for long-lived plants that are not allowed to reproduce within the gardens, constitute 'common garden experiments' led to many further studies, all carried out in the Munich botanical garden. Constantin, for example, cut off many species' branches to study their leaf-out under controlled conditions (in the greenhouse) and shaded branches outdoors by covering and uncovering them daily, which involved him living in the gardeners' building on

Fig. 8 Results of an experiment in the Munich botanical garden in which a single *Fagus sylvatica* branch was partially enclosed in a translucent bag (upper twig) or instead a light-tight bag (lower twig) every night from 1 January until 25 April 2014, with the bags being removed in the morning and placed back around the twig in the evening in such a way as to result in a constant 8-h-day length. The experiment revealed the absence of communication among the buds, and instead bud-specific responses to day length (Zohner and Renner 2015)

the garden grounds. The shading experiments led to the discovery that light perception happens at the level of the individual bud, with next to no communication among nearby buds on the same tree (Zohner and Renner 2015) (Fig. 8). And different from previous ideas, day length is a less useful signal of the arrival of spring further north than it is in south because at high latitudes, the fastest increase in daylength occurs long before the end of night frosts, which would kill any young leaves. Instead, northern trees rely on air temperature to optimize the timing of bud burst (Zohner et al. 2016, 2020). The more Constantin worked on botanical garden phenological data (also using those from gardens in North America and China), the more ideas we had about hypotheses to test. One idea was to test if introduced Asian species become invasive in North America because of their more flexible reaction to spring warming as had been suggested: The answer is no (Zohner et al. 2017; Zohner and Renner 2017). We also returned to the question that had bothered me since my own thesis, namely, the extent of synchrony within species, but now focusing on leaf-out in northern trees, not flowering in tropical species. We found that global warming reduces leaf-out and flowering synchrony among individuals (Zohner et al. 2018).

The collaboration with Constantin continues, currently focusing on the effects of climate warming on the onset and progression of leaf senescence, a topic of great interest because the total length of the growing season influences how much CO_2

long-lived plants will be able to take up from the atmosphere in the coming years (Zohner and Renner 2019; Zani et al. 2020). We are also studying the adaptive value of anthocyanin production in the fall, specifically the question why red leaf coloration is more frequent in Eastern North America (ENA) than anywhere else. Our hypothesis is that the reason is the higher light intensity experienced by ENA species during autumn compared to species growing at the same latitude in the other continents (Renner and Zohner 2019, 2020, 2022). As they say: More work is needed.

9 History of Botanical Collecting and New Ways to Achieve Stable Scientific Names

Most people become interested in the history of their field when they themselves become history. For me, however, the interest began already during my postdoc at the Smithsonian, inspired by my advisor, John Wurdack, and the historian Joseph Ewan, who encouraged me to write a history of botanical collecting in Eastern Ecuador (Renner 1993). Later, I became interested in Linnaeus's approach to naming new species (Renner 2016c), the story of J.V. Helfer, the earliest botanist to visit the Andaman Islands (Wolcott and Renner 2017), and A. v. Humboldt's treatment of his botanical data (ongoing research). The link between biological organisms, mental species concepts, and scientific names, which are metaphysical and inevitably context-dependent, is extremely difficult to stabilize. Since 1935, biologists concerned with naming species have agreed to link names and biological entities by selecting one specimen (or illustration) and labelling it as 'type' and then storing it in a publicly accessible collection, similar to how the standard meter used to be a physical piece of marble kept in Paris.

There are tens of thousands of labelled, by definition correctly identified, type specimens that provide 'yards sticks' for plant identification, and due to the digitization efforts in herbaria all over the World, there are many million high-resolution images of type and non-type specimens. These images will never be efficiently studied and used by human eyes and brains. Instead, the best way to take full advantage of this image resource is to apply machine-based image recognition using artificial intelligence (AI). In a pilot project in the Munich herbarium, we tested the application of computer vision for specimen identification with typical specimens of common German trees. The results showed that the computer did as well in matching names to species as did DNA barcoding (Unger et al. 2016).

The application of AI to specimen images and, equally important, to text on labels is a burgeoning field, although we are far from AI-based plant identification because many type specimens have overlapping, insect-eaten, untypical or simply poorly-preserved leaves. Single-locus-barcoding sequences, while providing a conceptual bridge from population genetics to systematics (Losos et al. 2013), also will not solve the above-mentioned problem of a stable application of names to plant specimens because most plant barcode marker sequences are too short and cannot reliably differentiate close relatives. Instead, what would help the stability of names in the future would be to permit nuclear genomes, or relevant parts thereof, as nomenclatural types if a systematist has these data, and the sequences meet certain standards. Traditional dried specimens, imaged and barcoded, could obviously also still be used, perhaps along with photos, videos, and other types of evidence. Continuing to strictly confine ourselves to dead museum specimens as permitted types does not seem a good strategy, especially if the goal is efficient conservation of species (Miralles et al. 2020; Renner 2021).

The required changes in the *Code of Nomenclature* for plants would be minor, and the zoological *Code of Nomenclature* already permits genomic data as nomenclatural types in certain situations. Changes to the *Codes* for prokaryotes and fungi are under discussion and have inspired my own thinking on this topic, as did Linnaeus's brilliant approach of relying on specimens, images, geography, and few-word statements, never descriptions, which he detested (Renner 2016c). If Linnaeus were alive and knew about type-based nomenclature, I am sure he would vote for permitting nuclear genomic data as types, especially for algae and fungi. In the future, we could stabilize the application of scientific names with a combination of curated genomes, specimens, images, and geography, with the data accessible worldwide 24/7/365.

Today, 31 January 2022, the Royal Botanic Gardens Kew made available a sequence data set of 353 nuclear orthologous genes for 9,833 genera of flowering plants and a few gymnosperms (https://treeoflife.kew.org/tree-of-life). Soon, the same set of nuclear genes will be available for all 13,600 angiosperm genera and 50% of the 8,000 fungal genera. An American-led international team has begun to produce a reference genome for each taxonomic family of eukaryotes (∼9,400 species), which they hope to achieve by 2025, followed by genomes for one representative of each genus (∼180,000 species) and then reference genomes for all ∼1.65 million known eukaryotic species (Lewin et al. 2022). Our *Codes of Nomenclature* need to keep pace by allowing for a larger role of genomic data in the naming of biological species.

10 Epilogue: The Increasing Role of Collaboration in Systematics and the Changing Position of Systematics Within Biology

Andreas Bresinsky (2014, p. 42) concluded his autobiographic sketch with a remark on how efforts to ensure phylogenetic coherence of higher-ranked groups with 'unique derived' characters have failed and been replaced by studies of diversification and trait changes within DNA-based clades. He speaks of a paradigmatic shift within the scientific branch of systematics. I could not say it better. Christian Körner (2021), in turn, ended his insightful sketch with remarks on what he learned from

Fig. 9 The official kick-off meeting of the DFG-funded priority program in ‘Taxon-Omics’ on 21 October 2017. The program’s steering committee consisted of (1) Dominik Begerow, (2) Elvira Hörandl, (3) Miguel Vences, and (4) myself. Also numbered are my former student Gudrun Kadereit (5), now the chair of systematic botany in Munich, another former student, Hanno Schaefer (6), now a professor in Munich, and former student and then-postdoc Constantin Zohner (7), now at the ETH in Zurich,

teaching, stressing that one should show feelings about the subject one teaches. I have done this to a fault and sometimes was offended when students didn’t listen; at least twice, I abruptly left huge lecture halls for that reason. Over my 33 years of academic teaching, I never had a doctoral student break off their studies and was invited to teach courses elsewhere, for example, in Senegal (Dakar 1993), Brazil (Pernambuco, Recife 1995), and Tanzania (Amani Research Station 2002). I, at least, enjoyed my teaching.

A clear shift over the past 40 years in the way systematists work is the far greater importance of collaboration. In the 1980s, systematic research, monographs, floras, and cladistics (still entirely morphology based) used to be done in isolation. With DNA data arrived lab work, bioinformatic pipelines, and a need, as well as the opportunity, to collaborate. Exactly in the spirit of collaboration among systematists – at least in the training of students – is a DFG priority program called ‘Taxon-Omics – New approaches for discovering and naming biodiversity’ that I organized with the mycologist Dominik Begerow, the botanist Elvira Hörandl, and the zoologist Miguel Vences and submitted in October 2015. We obtained funding for 25 projects (Fig. 9), and this German-wide program was so successful that it has been prolonged for another 5 years until 2025. Such collaborations across the 'tree of life,' enabled by a shared reliance on DNA-based phylogenies, would never have been possible when I started in systematics. Increased collaboration, also with biologists in other fields, has brought systematists out of their niche, given comparative biology a huge push, and resulted in a better integration of biodiversity studies within biology.

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Plants in Space: Novel Physiological Challenges and Adaptation Mechanisms

F. Javier Medina, Aránzazu Manzano, Khaled Y. Kamal, Malgorzata Ciska, and Raúl Herranz

Contents

Abstract Any space exploration initiative, such as the human presence in the Moon and Mars, must incorporate plants for life support. To enable space plant culture we need to understand how plants respond to extraterrestrial conditions, adapt to them, and compensate their deleterious effects at multiple levels. Gravity is a major difference between the terrestrial and the extraterrestrial environment. Gravitropism is the process of establishing a growth direction for plant organs according to the gravity vector. Gravity signals are sensed at specialized tissues by the motion of amyloplasts called statoliths and transduced to produce a cellular polarization capable of influencing the transport of auxin. Gravity alterations eventually result in changes in the lateral balance of auxin in the root, producing deviations of the growth direction. Under microgravity, auxin changes affect the root meristem causing increased cell proliferation and decreased cell growth. The nucleolus, the

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nuclear site of production of ribosomes, is a marker of this unbalance, which could alter plant development. At the molecular level, microgravity induces a reprogramming of gene expression that mostly affects plant defense systems against abiotic stresses, indicating that these categories of genes are involved in the adaptation to extraterrestrial habitats. Nevertheless, no specific genes for plant response to gravitational stress have been identified. Despite this stress, plants survive, developing until the adult stage and reproducing under microgravity conditions. A major research challenge is to identify environmental factors, such as light, which could interact, modulate, or balance the impact of gravity, contributing to the tolerance and survival of plants under spaceflight conditions. Understanding the crosstalk between light and gravity sensing will contribute to the success of the next generation agriculture in human settlements outside the Earth.

Keywords Auxin, Cell cycle, Gravitropism, Light signaling, Meristem, Microgravity, Nucleolus, Ribosome biogenesis, Spaceflight, Transcriptomics

1 Plants Are Needed for Space Exploration: Space Plant Biology

> *"Je ne sais pas si les mondes sont habités, et, comme je ne le sais pas, je vais y voir!"* ("I do not know if the worlds are inhabited, and, as I do not know that, I'll go there and see!")
> – Jules Verne, "From the Earth to the Moon" (1867).

This quote, from one of the most famous books by Jules Verne, which was written more than a century and a half ago, reflects an intense human dream, which at the same time is a major challenge for the humankind: to go out of our planet Earth and see how are "the other worlds", in particular, whether we, as living beings and, especially, as intelligent living beings, are alone in the Universe, or we have companions with whom we can interact. Science-fiction literature is full of stories talking about the relationships between humans and aliens, whether they are peaceful or hostile.

In 2019, the entire world has commemorated the 50th anniversary of the first human footprint on the surface of the Moon, and in 2020, the 20 years of the continuous presence of humans as crew members of the International Space Station (ISS). These commemorations have evidenced that space exploration is still considered by most people as a highly exciting and attractive challenge that, additionally, promotes the scientific and technological progress and significantly contributes to a better human life on Earth (Rai et al. 2016). Certainly, some relevant opinions have appeared expressing concerns and doubts, mostly focused on the high costs for citizens – tax payers that this enterprise entails in relation to its effective outcomes (Rinaldi 2016).

As a consequence of this social context, the leading countries of the world, in America, Europe, and Asia, are currently working to promote a manned mission, first to the Moon, and then to Mars. NASA has made public the "Artemis" program with the explicit goal of the landing of "the first woman and the next man" on the Moon by 2024. Europe, Japan, and Canada have expressed their support to this program as active partners. This objective is considered only as the first step in the run to a human settlement on the Mars surface in the decade of 2030s. On its side, the plans of the Chinese government, though less explicit, are not far from these objectives.

In this exciting story, life sciences must play a relevant role. The environment that space explorers will find in the spaceflight and in nearby planets is very different from Earth in many factors, which are not compatible with terrestrial life. Many of these adverse factors can be counteracted in spaceships, or in Martian or Lunar settlements, by developing specific habitats, but the astronauts, as living beings, and their accompanying living organisms, must adapt to grow and survive under the influence of a completely different gravity level and under radiation doses higher than those existing on the Earth. These environmental factors cannot be currently counteracted by physical means in an affordable and efficient manner. Thus, the main aim of space life science is to understand how the space environment and specifically altered gravity and radiation affect the morphology, physiology, and behavior of living organisms and to design countermeasures to enable terrestrial life, and particularly human life, to develop outside Earth. That is, how they perceive and respond to gravity and radiation and how they adapt to the space environment. This adaptation is the major objective of space life science despite an emerging opinion suggesting that terrestrial gravity should be provided to astronauts as part of their life support, together with, e.g., oxygen or temperature, in view of the severe damages induced by microgravity on the human physiology and the difficulties found in developing effective countermeasures (van Loon et al. 2020).

It is beyond doubt, in any case, that space explorers will need a constant and sustainable supply of oxygen, food, and vitamins, as well as the removal of their waste CO_2 and the regulation of the environmental moisture. Furthermore, their psychological wellbeing should not be neglected. Interestingly, all these resources can be supplied by a single – though varied and diverse – component, namely plants. Therefore, plants must be a key component of any bioregenerative life support system that can be designed for human space exploration.

The culture of plants in spaceflight, or on planets and satellites other than the Earth, such as the Moon or Mars, necessarily requires the creation of a "greenhouse" in which the plant is provided with the necessary environmental elements to enable its development. These elements include light, water, temperature, oxygen, CO_2, aeration, and nutrients, as well as microorganisms, in order to achieve a fully functional and sustainable environment for plants. These factors and conditions should be initially provided through import from the Earth, although, in the case of stable, mid- and long-duration settlements in the Moon or Mars, self-regenerative systems should be implemented to avoid the dependence of terrestrial supplies. For the case of water and light, native sources could be found and effectively used. Once these requirements are met, plant growth should be enabled in the presence of a gravity level different from the Earth value (near-zero *g* – microgravity – in orbit, 0.17 *g* for the Moon and 0.38 *g* for Mars) and of a certain level of cosmic radiations that escape from shielding devices. This requires that the specimens to be cultivated are adapted to grow and develop in the presence of these two environmental factors, under magnitudes significantly different from the Earth values. The development of technological countermeasures and the use of biological strategies that mitigate the unfavorable impact of gravity and radiation are complementary strategies to consider. These are the major current challenges that space plant biology needs to overcome.

2 Gravity Perception, Transduction, and Response

Gravity is an essential environmental factor for plant growth and development and, in addition, it has been a constant environmental factor throughout the entire history of the biological evolution in the planet Earth (Volkmann and Baluška 2006). The orientation of plant growth is driven by the unchanged direction and magnitude of the gravity vector, which is ultimately responsible for the growth of roots deep in the soil and the growth of stems in the upright direction, in which leaves can be maximally exposed to sunlight, for the most efficient accomplishment of photosynthesis. The process and mechanism by which plants sense and respond to the mechanical stimulus exerted by the gravity vector is termed gravitropism and involves the coordinated activity of different cell types and tissues.

Most of the knowledge acquired on the mechanisms of gravitropism has come from experiments involving the response of plants to a change in the growth orientation. As indicated, the plant growth direction is aligned with the gravity vector. The direction of this vector cannot be changed, but we can turn the plant and uncouple the alignment, such that the axis of the plant does not coincide anymore with the gravity vector. Therefore, the plant must reorient its growth, by re-establishing the alignment according to gravity. This produces a bending or curvature in the root and stem, and the biological mechanism by which this curvature is established illustrates the biological bases of the sensing, transduction and response to the gravity mechanosignal, that is, of the plant gravitropism.

The gravitropic response can be divided into four steps: gravity perception, transduction of the signal within the cell, transmission of the signal from the receptor cells to locations spatially different and separated in the plant, and the growth response (Swarup and Bennett 2018; Gadalla et al. 2018) (Fig. 1). Gravity perception takes place in specialized cells called statocytes. These cells have starch-containing plastids, statoliths, considered as gravity sensors, which sediment in the direction of the gravity vector. In the root, statocytes are located in the root cap (columella), whereas in the shoot, they are found in the endodermis. A change in the gravity direction is sensed by the displacement of statoliths, which re-sediment according to the new direction of the gravity vector (Fig. 1). The discrimination of the precision of the system, in terms of the threshold angle of inclination capable of triggering a response, and of the response time, is an important issue. It could be

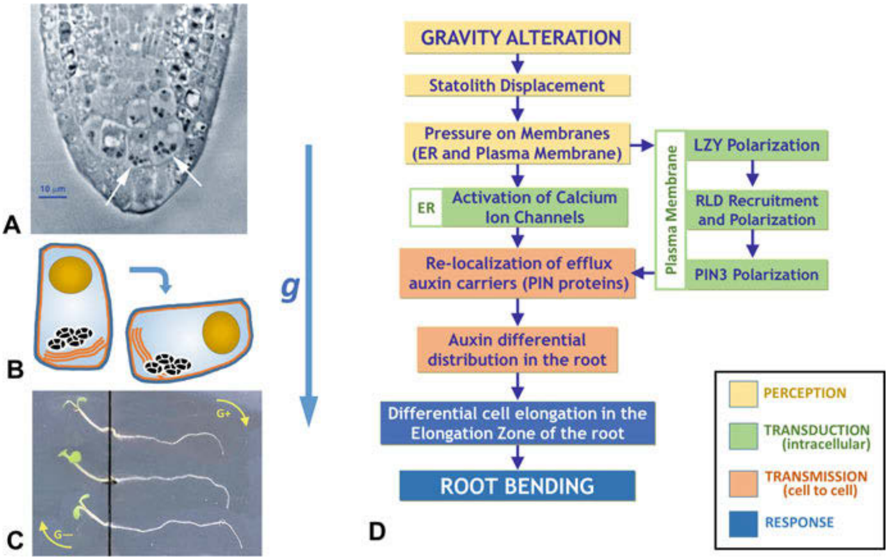


Fig. 1 Root gravitropism: gravity sensing and response in roots. (**a**) Section of an *Arabidopsis thaliana* root tip observed unstained, with phase contrast microscopy. Statoliths (arrows) appear sedimented in the bottom part of columella cells, according to the gravity vector *(g)*. (**b**) A turn of 90° with respect to the gravity vector produces a displacement of statoliths toward the side of the cell that is now at the bottom. (**c**) The results of the turn of 90° in seedlings is the bending of the root and hypocotyl, to reorient their growth direction according to the gravity vector. (**d**) Sequence of events in the gravity sensing and response. The gravitropic signal is sensed and transduced in columella cells, and it is then transmitted throughout the root to produce a change in the lateral distribution of auxin, which results in root bending due to the differential cell elongation occurring in a specific zone of the root

thought that the density of the intracellular milieu and the interactions of amyloplasts with endoplasmic reticulum (ER) and cytoskeleton would restrict the flow of the granules, which would cause the granular system to stop working below a critical angle. However, the statoliths offer surprising precision, as demonstrated by the response of the plant aerial organs to a very weak tilt, allowing the maintenance of the vertical posture of the plant (Moulia et al. 2019). Actually, it has been shown that, in response to an inclination, statoliths do not behave like a classic granular medium, but they move and flow in the cell regardless of the angle of inclination. Like a liquid, the surface of the statolith deposits always recovers horizontally. Using a biomimetic system, consisting of microbeads arranged inside artificial cells of the same size, it was concluded that the joint fluidity of the statoliths derives from the movement of each of them separately. The cellular molecular motors (actin-myosin network) constantly shake them, allowing them not to get stuck and giving to the system, as a whole, properties close to those of a liquid (Bérut et al. 2018). This behavior is essential for the plant, since it allows it to react to the smallest inclinations.

Once the gravitropic signal is sensed by statolith movement, it has to be transduced to produce a physiological response. Although the chain of events in this transduction is not totally and precisely understood, there is experimental evidence that the physical connection of statoliths with the cytoskeleton (especially with actin microfilaments) and with membranes (ER and plasma membrane) is involved in this process. The actin cytoskeleton participates in the statolith motion as part of its general function in the cell as a molecular motor for the intracellular transport of organelles (Kadota et al. 2009). However, an additional role of actin microfilaments in the transmission of the tensions generated by sedimenting statoliths to mechanoreceptors on cell membranes (tensegrity) was reported (Yoder et al. 2001). In apparent contradiction with these roles of actin, experiments involving the treatment of plants with actin inhibitors resulted in an enhancement of the gravitropic response (Yamamoto and Kiss 2002). Actually, actin microfilaments act, at the same time, transmitting the gravitropic stimulus to ER membranes and relieving in part the compressive forces of amyloplasts on membranes. Therefore, a disruption of microfilaments would increase the compressive forces on ER, eventually enhancing gravity sensing and signaling (Leitz et al. 2009). Thus, although an intact actin cystoskeleton is not strictly required for gravitropic signaling and response, it indeed plays a role in the fine-tuning of the early steps of the gravitropic process, as it was additionally suggested by genetic studies indicating the involvement of specific proteins in the interaction between statoliths and actin (Blancaflor 2013).

The statolith motion induces a pressure exerted by these organelles on cell membranes, specifically on ER, which causes membrane deformations, as it has been visualized by electron tomography (Leitz et al. 2009). The consequence of this deformation is the opening of membrane-localized mechanosensitive Ca^{2+} channels that create a Ca^{2+} signal in the cytoplasm (Toyota and Gilroy 2013). This results in a fast alkalization of the cytoplasm of statocytes, which correlates with a change in cellular polarity involving redistribution of auxin efflux carriers (PINs). This correlation of pH change and relocation of PIN proteins is supported by the finding that mutants defective in alkalization of columella cells do not show PIN relocation (Baldwin et al. 2013). The resulting ion current can trigger further signaling cascades (Fig. 1).

Therefore, despite the gaps still existing in our understanding of the precise mechanisms of gravity sensing and signal transduction in the root, the process can be summarized as a message sent by collumella cells to the elongation zone, affecting auxin lateral distribution (Baldwin et al. 2013). The gravitropic signal, perceived by the new location of statoliths, ultimately produces the asymmetric redistribution of the plant hormone, auxin, as a result of auxin signaling events that depend on the coordinated activity of auxin influx (AUX1) and efflux carriers (PIN2, PIN3) within the root apex (Bennett et al. 1996; Friml et al. 2002). Additional support for this mechanism comes from the fact that the *aux1* mutant is totally agravitropic, whereas mutations in various PIN protein genes confer reduced gravicompetence. PIN3 and PIN7, located in the columella cells, change their distribution within minutes after the gravitropic stimuli, and PIN2 transports auxin through epidermis toward the root elongation zone. The mechanism results in an increased auxin concentration at the lower side of rotated roots that inhibits cell growth in the lower side of the elongation zone, causing a curvature of the root to align itself with the gravity vector (Baldwin et al. 2013). This root bending can be inhibited by a treatment with NPA, an inhibitor of the auxin polar transport (Rashotte et al. 2001).

Recent studies have revealed a fundamental role of the LAZY1 protein family in early phases of gravitropic signal transduction, by acting as intermediates between the relocation of amyloplasts and the gravity-induced change in the directional auxin transport (Nakamura et al. 2019). From the six genes of this family, four of them *(LZY1* to *LZY4)* are expressed in gravity-sensing cells. The involvement of this gene family in gravity signaling was shown in a transcriptomic study carried out in two *Arabidopsis* mutants defective in the gravitropic response. Three members of the family, namely *LZY1, LZY2*, and *LZY3* were identified and characterized. In the triple mutant, the amyloplast relocation was found unaltered, but the formation of the asymmetric PIN3 distribution and auxin flow were reversed. Phenotypically, the mutant plants showed alterations in the growth angle of lateral shoots and roots. Thus, LAZY proteins were suggested to play a key role in controlling lateral auxin flow after the reorientation of statocytes (Taniguchi et al. 2017). The expression pattern of all six genes of the family was analyzed in specific constructs harboring reporter genes. Analysis of single and multiple mutant lines reveals that single mutants show only mild alterations in the growth direction of lateral roots, which are enhanced in the *lzy2 lzy3* double mutant, and the *lzy2 lzy3 lzy4* triple mutant displays reversed root gravitropism, associated to a reversion of the asymmetric auxin distribution (Yoshihara and Spalding 2017). More recently, a model of gravity signaling has been proposed, involving the participation of LZY proteins and their identified interactors, RCC1-like domain (RLD) proteins, in the modulation of auxin flow (Furutani et al. 2020). RLD proteins were shown to act in controlling the abundance and localization of the PIN3 protein. Plasma membrane is likely to be the site of action of LZY proteins in statocytes. Gravistimulation, and the subsequent amyloplasts relocation, induces polarization of LZY3 localization in the direction of gravity in the plasma membrane of columella cells. This polarized LZY recruits RLD proteins from the cytoplasm to the plasma membrane, and RLD would modify PIN3 trafficking, leading to asymmetric auxin flow (Furutani et al. 2020). Therefore, LZY proteins are acting downstream statoliths displacement signal, but upstream auxin transport (Fig. 1).

As it can be inferred from the experiments quoted in the preceding paragraphs, our knowledge of the mechanisms of plant gravity sensing and gravitropic response largely comes from studies performed after induction of a change in the growth direction of the plant with respect to the gravity vector, usually involving a rotation of the seedling or the plant. However, what happens if a gravitropic signal is not sensed by the plant or seedling, because the gravity vector is absent, as it occurs in weightlessness, or under microgravity conditions, e.g. on board of spaceships? And what is the response of the plant to a change in the gravity vector that does not affect to its direction, but to its magnitude, as it occurs in potential habitats in which gravity force is a fraction of the terrestrial value, such as the Moon or Mars? Certainly, we know significantly less on the plant response in these conditions, simply because experimentation is more constrained. This experimentation was performed either in real microgravity (spaceflight) or using ground-based facilities for simulated microgravity (or fractional gravity), such as the clinostat or the random positioning machine (RPM).

If the morphogenesis of plants under the influence of the terrestrial gravity vector is called “gravimorphogenesis,” the mechanisms of response to an environment without the influence of a defined gravity vector (real or simulated microgravity) constitute the so-called automorphogenesis, a process that began to be known and studied as early as in the nineteenth century, using the most classic clinostats. Classical studies carried out in these devices described spontaneous curvatures of roots followed by straight root elongations in random directions (reviewed by Hoson and Soga 2003).

In real microgravity, different studies performed in space experiments also revealed automorphogenesis, but both random and non-random growth directions of roots grown in microgravity were described in different experiments. An experiment on the growth of rice roots in spaceflight showed that in the early phase of growth, most of them elongated in a constant direction, forming a constant angle of about 55° relative to the seed axis, but later the roots grew randomly in various directions, including away from the culture medium (Hoson et al. 2003). In a more recent experiment carried out in the International Space Station (ISS), lentil roots initially curved strongly away from the cotyledons and then slowly straightened out forming a relatively constant angle (Driss-Ecole et al. 2008). A specific phenomenon, termed root skewing was repeatedly observed in seedlings growing in spaceflight (Millar et al. 2011; Paul et al. 2012a). It was defined, under normal ground gravity conditions, as the deviation of the root growth from the gravity direction, caused by a combination of factors, such as the interaction of gravity and touch to a slanted impenetrable medium and the inherent tendency of the root tip to circumnutate with a fixed handedness (Roy and Bassham 2014). The molecular mechanisms underlying such growth phenomenon, and especially its frequent occurrence under microgravity conditions, remain unresolved, although various genes and factors have been proposed to regulate root skewing, involving polar auxin transport and cytoskeletal dynamics (Nakashima et al. 2014; Roy and Bassham 2014). A recent study in the ISS, using two mutants of skewing behavior, affecting different cellular functions, concluded that genes related to skewing could play a prominent role in plant spaceflight adaptation (see later in this chapter) (Califar et al. 2020).

The kinetics of the movement of statoliths in microgravity conditions was studied in spaceflight in an experiment combining 1 *g* centrifugal acceleration and direct exposure to weightlessness. Amyloplasts were grouped at the distal pole of the statocytes by a centrifuge pulse and then placed in microgravity for increasing periods of time (13, 29, 46, or 122 min) and chemically fixed. Electron microscopical observations showed a gradual displacement of statoliths toward the proximal pole, but this movement was stopped by the nucleus. This position of statoliths, grouped beneath the nucleus, was similar to that observed in roots grown continuously in microgravity. Treatment with cytochalasin D demonstrated the involvement of the actin microfilaments in the statolith displacement (Driss-École et al. 2000). A similar behavior was observed in simulated microgravity, using the RPM (Kraft et al. 2000).

As a consequence of the changes in the mechanisms of gravity sensing and response in weightlessness, a significant inhibition of the auxin polar transport was reported in early spaceflight experiments (Ueda et al. 1999). Otherwise, when auxin transport was experimentally inhibited, the gravitropic response was suppressed (Muday and Haworth 1994). In a study using different systems of microgravity simulation, namely magnetic levitation and the RPM, the pattern of auxin distribution in the root tip under simulated microgravity, visualized with the reporter gene construction DR5::GUS, was shown to correspond to the inhibition of the auxin polar transport (Herranz et al. 2014). However, in a more recent experiment performed in space, the distribution of auxin in the root was shown to display a "vertical" pattern, similar to the pattern of roots grown under control ground gravity, even though the roots showed a disoriented growth, including numerous bends, coils, and skews (Ferl and Paul 2016). The authors conclude that the auxin transport through the root and the balance of auxin distribution in the root would be independent from gravity sensing. Actually, more research is necessary to explain these findings in the context of the current models of relationships between statolith movement, auxin transport, and root growth direction.

3 Auxin and Meristems: Effects of Microgravity on Meristematic Cells

Auxin fulfills multiple roles in the regulation of plant growth and development, acting in many steps and processes. In the root, auxin establishes the root architecture through its function in initiation and emergence of lateral roots, patterning of the root meristem, and cell expansion in the elongation zone. The configuration of the auxin polar transport throughout the different zones of the root is essential for determining root morphology and anatomy. Under standard environmental conditions for plant growth, in absence of any stress, it has been observed that the auxin maximum accumulation occurs in the proximity of quiescent center in the root tip (Vanneste and Friml 2009).

The function of auxin in plant growth and development is based on the role of this phytohormone in the regulation of organogenesis. This involves the controlled production of new cells in meristems, which are specialized zones of the plant containing a permanent population of undifferentiated totipotent cells whose function is to grow and divide. Thus, regulation of cell proliferation and growth, with further cell expansion and differentiation, in meristems is the basis of plant development. Auxin promotes cell division and drives meristem maintenance, and also plays an important role in the establishment of cellular patterning, according to specific threshold concentrations and cell- or tissue-specific responses

(Perrot-Rechenmann 2010). More precisely, auxin is necessary but not sufficient to stimulate cell division in cultured cells or plant tissues because the presence of cytokinin is also required (Inzé and De Veylder 2006). Conversely, addition of auxin to arrested cells after deprivation of auxin leads to restoration of cell division (Perrot-Rechenmann 2010). The existence of meristems, their functions and mechanisms in relation to differentiation, and their ability of supplying new differentiated cells in any moment of the life of the plant is a basis of the plant plasticity. By means of this plasticity, plants counteract their sessile condition and may adapt to a wide range of environmental changes and conditions.

Unsurprisingly, auxin plays an important role during abiotic stress-induced changes in the root. Through the creation of local auxin maxima and minima, the balance between cell division and cell differentiation, the rate of cell elongation and the emergence of lateral roots can be modulated in response to environmental signals. By these mechanisms, root architecture is ultimately modified in dependence of external stimuli, and auxin becomes an essential player in the plant acclimation to changes in the environmental conditions (Korver et al. 2018). The plant response to gravity changes is not an exception.

3.1 Effects of Microgravity on the Cell Cycle

The influence of environmental gravity on meristematic cell functions, mediated by changes in the levels and distribution of auxin, is only partially known. A relatively small number of experiments have approached this topic, in spaceflight and using microgravity simulation devices on Earth. Most of these experiments were focused on the cell cycle regulation (see Herranz and Medina 2014). Early pioneering studies on spaceflight, using lentils as model species, reported changes in mitotic index of roots grown in microgravity (Darbelley et al. 1986; Driss-École et al. 1994). From data on this parameter it was difficult to draw firm conclusions on the factors and mechanisms affected, but it clearly pointed out that alterations of cell cycle caused by the space environment may occur. Further experiments in real and simulated microgravity showed changes in the proportion of cells in different cell cycle phases, measured by densitometric analysis of nuclear DNA content of root meristematic cells, suggesting that the regulation of the cell cycle progression is somehow modulated by gravity (Legué et al. 1996; Yu et al. 1999).

The first European experiment on plant biology on board the ISS (the “Root” experiment) revealed that one of the most relevant effects of altered gravity was the dissociation of cell proliferation from cell growth in seedling root meristems. A remarkable increase of the cell division rate and a decrease of cell growth were observed. The strict coordination of the rates of these two fundamental processes that characterize meristematic cells was called “meristematic competence” (Mizukami 2001). Consequently, exposure of seedlings to microgravity produces a deep alteration of the normal function of cells of the root apical meristem (Matía et al. 2010). Subsequent experiments performed with seedlings on Earth using different ground facilities for simulation of altered gravity conditions showed similar trends as the spaceflight experiments. Specifically, the expression levels of cyclin B1 showed a significant decrease in simulated microgravity, as a sign of alterations in the regulation of cell cycle progression (Manzano et al. 2013; Boucheron-Dubuisson et al. 2016). Additional experiments with callus cell cultures in real and simulated microgravity, using genomic and proteomic methods, also demonstrated alterations in the expression of genes and in the levels of proteins involved in cell cycle regulation (Fengler et al. 2015; Manzano et al. 2012; Barjaktarovic et al. 2009; Paul et al. 2012b).

More recent studies have used an *Arabidopsis in vitro* suspension culture immobilized in agar and incubated in the RPM for simulation of microgravity. A suspension culture is a powerful tool in plant cell cycle studies and has the additional advantage of the possibility of synchronizing cells for their progression in the cell cycle (Menges and Murray 2006). This study was complemented with simulation of the Mars gravity (0.38 *g*) and hypergravity (2 *g*). Different times of exposure to altered gravity were tested. Whereas 3 and 14 h of growth in experimental conditions produced only mild effects, 24 h of exposure, the approximate duration of an entire cell cycle, produced the most relevant alterations. The most intense effects were found for simulated reduced gravity, whereas hypergravity produced milder effects. Cell proliferation and growth were uncoupled under simulated reduced gravity, similarly as the results obtained in root meristematic cells from seedlings grown in real or simulated microgravity. Alterations in the duration of cell cycle phases were reported, as well as in the tested cell cycle regulators, both affecting the protein levels and the rate of gene transcription (Kamal et al. 2018).

A further study detected variations in the duration of cell cycle phases by flow cytometry, after synchronizing cells in their cell cycle progression by aphidicolin (Menges and Murray 2006). Cell cycle acceleration was demonstrated in cells grown in simulated microgravity, particularly at the cell cycle period comprising G2 and M (mitosis) phases. This period showed a remarkable reduction in time with respect to the 1 *g* control, outside the RPM. Oppositely, the duration of the G1 period was slightly longer in cells grown in the RPM than in control cells. Alterations in the so-called core cell cycle regulators (Menges et al. 2005), as well as in factors of epigenetic modifications were found in specific cell cycle phases using flow cytometry for protein level evaluation, and qPCR for gene expression. Most of the factors acting at the G2/M cell cycle checkpoint appeared downregulated under simulated microgravity, whereas most of the factors acting at the G1/S checkpoint showed upregulation. Furthermore, in these conditions, nuclear transcription by RNA polymerase II was depleted, while condensed chromatin increased. This was related to the epigenetic regulation of gene expression, including increased DNA methylation and depleted histone acetylation. Therefore, G2/M checkpoint disruption was a significant effect of altered gravity, as well as chromatin remodeling. An additional consequence is the existence of mechanosensors in individual cells, independent from the mechanisms of gravity perception acting on plants, which involve specialized (“professional”) cells (Kamal et al. 2019a). This accelerated cell cycle with a reduced subpopulation of cells in G2 and M phases reconciles the apparent paradox of an increased cell proliferation rate in microgravity (Matía et al. 2010) with a reduced expression of the *CycB1* gene as a marker of G2/M transition (Boucheron-Dubuisson et al. 2016; Manzano et al. 2013).

In a global transcriptomic analysis using the same *Arabidopsis in vitro* cell culture the overall transcriptomic response of cell cultures exposed to simulated microgravity, and, particularly, the differential effects on G1 and G2/M subpopulations of cells were investigated in order to provide new insights into the stress pathways involved in the response to altered gravity conditions. Separate analyses were carried out for different cell populations, namely G2/M- and G1-phase-enriched and an asynchronous culture sample. The gene ontology groups showing the most conspicuous differential expression were cell proliferation, energy/redox and stress responses, plus unknown biological processes. Globally, simulated microgravity produced overall expression inhibition in the three cell populations but the G2/M-phase-enriched cells showed the highest number of downregulated genes and stress response components changed dramatically from G2/M to the G1 subpopulation, suggesting a differential adaptive response to simulated microgravity through the cell cycle (Kamal et al. 2019b). The adaptation of cell cycle regulation, using both known stress mechanisms and unknown function genes, may cope with reduced gravity as a novel evolutionary environment.

3.2 *Effects of Microgravity on Ribosome Biogenesis and the Morphofunctional Organization of the Nucleolus*

Other than the rate of cell proliferation, the second component of meristematic competence is cell growth. The intuitive concept of cell growth is the increase in size of a cell, but, actually, distinct biological processes may eventually result in an enlarged cell and not all of them should strictly be termed as "cell growth". This concept should be only attributed to the increase in size of proliferating cells, which occurs throughout the interphase of the cell cycle, resulting in the increase in total cellular content of nucleic acids and proteins. No vacuolization is associated to cell growth. In contrast, the process of "cell expansion", also involving the cell enlargement, is associated to the process of differentiation, from a totipotent to a specialized cell and usually involves vacuolization and DNA endoreplication (Perrot-Rechenmann 2010).

Therefore, in highly proliferating cells, such as those of an *in vitro* suspension culture or meristematic tissues of plants, cell growth involves the production of proteins in order to overcome a biomass threshold (or cell size threshold) compatible with the viable size and biomass of daughter cells after mitosis (Doerner 2008). This is the reason for the need of a strict coordination between the rates of cell growth and cell division in proliferating cells, which defines meristematic competence (Mizukami 2001). Consequently, cell growth is largely determined by the activity of biogenesis of ribosomes, the cytoplasmic factories of proteins, which occurs in a prominent nuclear domain, the nucleolus (Baserga 2007; Bernstein and Baserga 2004; Bernstein et al. 2007).

Ribosome biogenesis is a complex multi-step process. It starts with the transcription of ribosomal genes, which are present in multiple copies of the 45S rRNA transcription unit, arranged in tandem and clustered in one or more specific chromosomal region(s) called nucleolar organizer regions (NOR). This is followed by the cleavage of 45S pre-rRNA to produce the mature rRNAs, which form cytoplasmic ribosomes in association with 5S rRNA and ribosomal proteins (Sáez-Vásquez and Medina 2008).

In addition to ribosomal proteins, hundreds of non-ribosomal proteins, or nucleolar proteins, play specialized roles either as enzymes, or regulating the rate of ribosome production in concert with snoRNAs, or even assuring the structural arrangement of the process (Sáez-Vásquez and Delseny 2019). Some of them interact with factors or mechanisms controlling cell proliferation and cell cycle progression (Medina and González-Camacho 2003), thus linking these crucial cellular processes with ribosome biogenesis and assuring meristematic competence.

Among nucleolar proteins, the multifunctional nucleolin is the most abundant protein of the nucleolus in actively proliferating cells, where it plays a key role in different steps, including rRNA gene transcription, processing of pre-rRNA, and assembly and nucleocytoplasmic transport of ribosome particles. It has also been implicated in other functions, with or without collateral relationship with ribosome biogenesis. For these functional roles, nucleolin activity is controlled at transcriptional, post-transcriptional, and post-translational levels during cell growth and differentiation, as well as in response to cellular stresses. In particular, nucleolin phosphorylation is mediated by kinases involved in cell cycle control (Durut and Sáez-Vásquez 2015; Tajrishi et al. 2011; Medina et al. 2010). While animal and yeast genomes encode a single nucleolin gene, plants show gene multiplicity. In *A. thaliana*, two genes encoding nucleolin proteins have been described: *NUC1* and *NUC2* (Pontvianne et al. 2007). The *NUC1* gene is highly and ubiquitously expressed in normal growth conditions. *NUC2* is a functional protein-coding gene developmentally controlled in most plant tissues and organs, and it contains several cis-acting elements related to biotic and abiotic stress responses (Durut et al. 2014). NUC1 and NUC2 proteins localize in nucleolus. Specifically, NUC1 localizes preferentially in the dense fibrillar component (DFC) and NUC2 colocalizes with peri-nucleolar chromatin, but only NUC1 is able to assist nucleosome remodeling *in vitro* (Durut and Sáez-Vásquez 2015; Medina et al. 2010; Pontvianne et al. 2007; Durut et al. 2014).

Fibrillarin is another nucleolar protein, which functions in the first pre-rRNA processing step, guided by pre-rRNA methylation, in association with snoRNAs and other nucleolar proteins, including nucleolin. Both fibrillarin and nucleolin have been reported to be co-purified in a snoRNP complex and to mostly share their *in situ* localization in the DFC of the nucleolus (Sáez-Vásquez et al. 2004; Medina and González-Camacho 2003).

As a fundamental cellular process, regulation of ribosome biogenesis is modulated by the environmental conditions and coordinated with other cellular processes, such as cell division and differentiation. Therefore, this process is a major and very useful stress sensor, and many stressors such as DNA damage, temperature changes, hypoxia, osmotic stress, oxidative stress, viral infection, and lack of nutrients have been shown to dramatically alter the rate of ribosome production (Boulon et al. 2010; Mayer and Grummt 2005; Kalinina et al. 2018). Gravity alteration is also capable of producing deep changes in this parameter. This is particularly relevant in highly proliferating cells, such as those constituting the plant meristems and *in vitro* cell cultures. As indicated above, in these cells the rate of ribosome biogenesis is a marker of cell growth and hence of the status of meristematic competence, in concert with the rate of cell division. A significant decrease in the efficiency of the biosynthetic machinery of ribosomes has been detected under real and simulated microgravity in plant proliferating cells, either *in planta* (root meristems from seedlings) or in culture *in vitro.* Nucleolar proteins, particularly nucleolin and fibrillarin have been chosen as reliable markers to detect and evaluate these alterations. The first results were obtained in a pioneering experiment in the ISS, already mentioned, in which the levels of nucleolin were quantified *in situ* by ultrastructural immunolabeling in meristematic cells. A significant reduction of the nucleolin labeling in nucleoli, of around one third of the levels of control 1 *g* samples, was found in samples grown in spaceflight, and also in samples grown under simulated microgravity conditions (Matía et al. 2010). These results were confirmed in successive experiments, using alternative methods of microgravity simulation (Boucheron-Dubuisson et al. 2016; Manzano et al. 2013). The exposure to simulated microgravity of the *nuc1* mutant, characterized by a disorganized nucleolus in ground gravity conditions, produced an intensified nucleolar disorganization and the appearance of nucleolar particles identified as corresponding to wrongly or incompletely processed preribosomal precursors (Boucheron-Dubuisson et al. 2016). A recent detailed analysis of pre-rRNA processing steps in seedlings grown in simulated microgravity has confirmed these alterations (Manzano et al. 2020b).

A deeper study on the effects of altered gravity on ribosome biogenesis through the alterations found on nucleolar proteins was carried out using an *in vitro* plant cell culture exposed to simulated microgravity. This experimental model offered some advantages with respect to meristems. Firstly, no limitations in the amount of biological material, necessary for molecular biology experiments, and, secondly, the possibility of using cell populations synchronized in their progression through the cell cycle. The decrease in the levels of fibrillarin and nucleolin caused by simulated microgravity was confirmed using immunolabeling quantified by flow cytometry and a downregulation in the expression of the *NUC1* gene was revealed by qPCR in cells grown in the microgravity simulator (Fig. 2) (Kamal et al. 2018, 2019a).

An additional reliable and useful estimation of the effects of gravity alteration on ribosome biogenesis in relation to cell proliferation and cell cycle was obtained by a thorough analysis of the structure of the nucleolus. The nucleolus is a polymorphic structure, extremely sensitive to functional changes involving different rates of ribosome production, such as those occurring throughout the different periods of the cell cycle in proliferating cells. Thus, structural features of the nucleolus are a

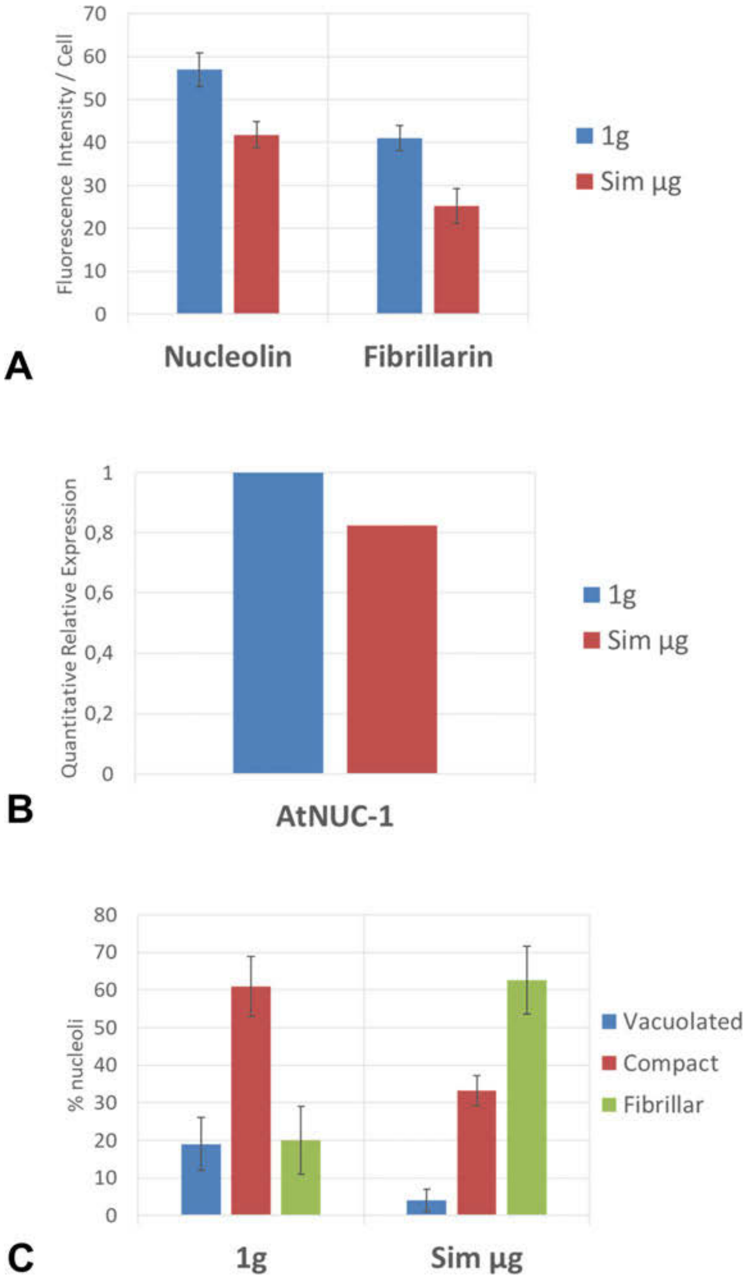


Fig. 2 Quantification of different markers of nucleolar activity and ribosome biogenesis after exposure of an *A. thaliana* cell culture to simulated microgravity. (**a**) Levels of two nucleolar proteins, nucleolin and fibrillarin, estimated by flow cytometry. (**b**) Expression of the nucleolin-1 (*Nuc1)* gene, estimated by qPCR. (**c**) Distribution of the different nucleolar types with functional significance in the cell culture. In all cases, the exposure to simulated microgravity results in a significant depletion of the nucleolar activity and, consequently of the rate of production of ribosomes

reliable marker of the functional state of the cell and, in particular, of the cell cycle phases (Sáez-Vásquez and Medina 2008). Moreover, the various effects on ribosome production and cell growth induced by the different types of cellular stresses are often accompanied by significant changes in the structural organization and distribution of the nucleolar components (Raska et al. 2006; Srivastava and Pollard 1999). These findings can be used as an efficient tool for the study of the cellular effects of environmental gravity alteration.

Based on previous knowledge on the molecular architecture of the nucleolus and on the distribution of nucleolar subcomponents in dependence of the activity of ribosome biogenesis in plant meristematic cells (Medina et al. 2000; González-Camacho and Medina 2006; Sáez-Vásquez and Medina 2008), three nucleolar structural types or models were defined in *Arabidopsis* proliferating cells with the purpose of that they could serve as indicators of different functional states of the cell, in relation to potential cellular stresses and also to cell cycle phases. These models, identified under the transmission electron microscope were called, according to their morphology, as “vacuolated,” “compact,” and “fibrillar,” arranged from the most to the less active in ribosome production (Fig. 3). To clarify nomenclature, nucleolar “vacuoles” refer to large and clear intranucleolar spaces that contain granules similar to the granular component (GC), that are made of nearly mature preribosomal particles. Their relationship with cytoplasmic vacuoles is only structural resemblance, and not functional analogy. Vacuolated nucleoli were characterized four decades ago in meristematic cells, as the expression of highly active nucleoli (Moreno Díaz de la Espina et al. 1980).

Moreover, a correlation between the structural features and the size of the nucleolus exists, so that the structural arrangement also corresponds to the size arrangement, form larger to smaller. Based on these features, these models can be easily identified on 2 μm semithin sections at the light microscope level (Manzano et al. 2016). These nucleolar models have a potential applicability as environmental stress sensors or as pathological markers.

The relative abundance of each of these nucleolar types, as an estimation of the status of the rate of ribosome biogenesis, and hence of cell growth, was statistically assessed in different conditions of gravity in an *in vitro Arabidopsis* cell callus culture exposed to magnetic levitation as a method of microgravity simulation. Samples exposed to simulated microgravity for 200 min showed a significant decrease in the nucleolar “active” type and an increase in the “inactive” type, compared to 1 *g* controls. The functional significance of the structural data was validated by several different complementary cellular and molecular indicators (Manzano et al. 2016). These results were confirmed using a suspension cell culture (Kamal et al. 2018) (Fig. 2c).

Otherwise, as previously indicated, and described in several plant model systems, a link exists between nucleolar morphology and cell cycle phases (González-Camacho and Medina 2006). The use of an *Arabidopsis* suspension cell culture synchronized in the cell cycle progression with a pulse of aphidicolin (Menges and Murray 2006) has allowed the unequivocal association between cell cycle phases and nucleolar models at the ultrastructural level. The same analysis performed after

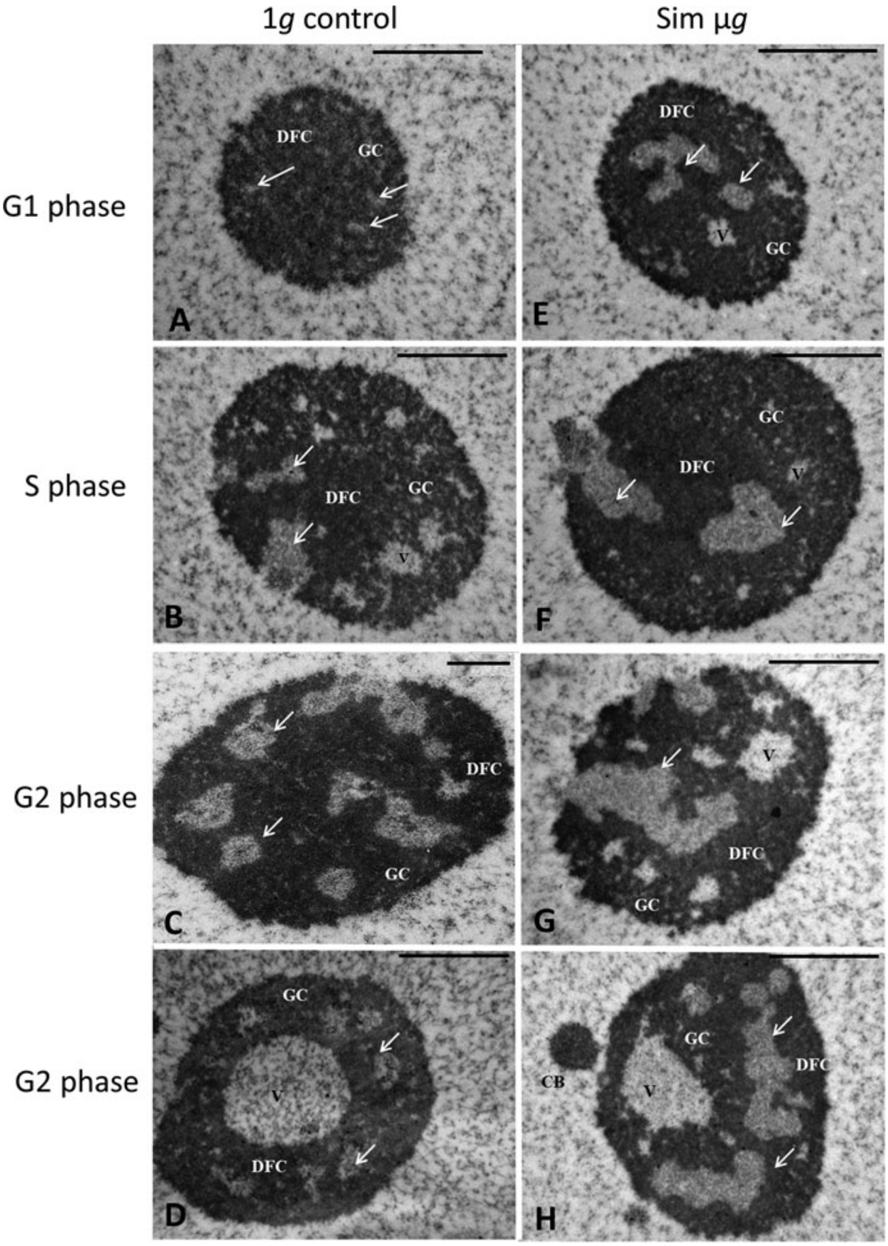


Fig. 3 Ultrastructural study of the nucleolus throughout the different phases of the cell cycle in *A. thaliana* cultured cells, synchronized with aphidicolin and exposed to simulated microgravity, compared to the same cell culture grown under 1 *g* control conditions. The structural and morphometric features of the nucleolus show dramatic changes in the different cell cycle phases, even under control conditions. In G1, the nucleolus is small, compact, and mostly fibrillar in structure (**a**). In S-phase, the size increases, granular component (GC) appears surrounding the dense fibrillar component (DFC) and several fibrillar centers (arrows) distribute scattered throughout the DFC (**b**). In G2, the nucleolus reaches the largest size and two structural types can be identified, namely

the incubation of the synchronized cell culture in a microgravity simulator has provided valuable information of the morphofunctional changes induced by altered gravity on the nucleolus in each phase of the cell cycle (Fig. 3). In parallel, under the light microscope, the quantitative distribution of the nucleolar types in the different phases was statistically assessed.

Under normal ground gravity, in the G1 phase, nucleoli appeared compacted, constituted practically only by dense fibrillar component (DFC) (Fig. 3a). Interestingly, G1 nucleoli did not show any type of nucleolar "vacuolar" space in *Arabidopsis*, such that it was observed in onion (González-Camacho and Medina 2006). G1 compact nucleoli were the smallest among the different nucleolar models which characterized cell cycle phases. This nucleolar structure and organization in G1 phase was altered by simulated microgravity; under these conditions, small nucleolar vacuoles appeared and fibrillar centers (FCs) slightly enlarged. Furthermore, this gravitational alteration did not produce significant differences in the nucleolar size (Fig. 3e).

In the S phase, at 1 *g*, the nucleolus was double-sized, compared to the G1 phase. This increment was accompanied by conspicuous changes in the ultrastructural features. The granular component (GC) appeared in the S phase as an abundant component, giving account of 60% of the nucleolar volume and clearly segregated from DFC, which was reduced in proportion. Small vacuolar areas and a few enlarged FCs were observed (Fig. 3b). S-phase-associated nucleolus structure was altered by simulated microgravity. Although the nucleolar size was not significantly affected, the distribution of the nucleolar components was different from the 1 *g* control. The abundance of DFC significantly increased, GC diminished, and the size of FCs was observed to increment, appearing interconnected in ultrathin sections (Fig. 3f).

Finally, in the G2 phase, two types of the nucleolar models could be distinguished in samples grown at the 1 *g* terrestrial gravity, depending on the presence or the absence of a large central nucleolar vacuole. The "non-vacuolated" model was similar in many aspects to the one in S phase; differences were the larger size, the intermingled distribution of DFC and GC and the multiplicity of smaller FCs (Fig. 3c). The "vacuolated" model shows a large central space, or "vacuole" containing loosely distributed granules, surrounded by a cortex formed by intermingled DFC and GC and small FCs (Fig. 3d). Simulated microgravity produced substantial effects in the nucleolar structure during G2 phase. Both models showed a larger proportion of DFC and FCs, these latter appearing conspicuously enlarged, at the expenses of GC. Furthermore, G2 nucleoli under simulated

Fig. 3 (continued) compact (**c**), with many small fibriilar centers, and vacuolated (**d**), with a large central space, or "vacuole" containing granules. In both types, DFC and GC appear intermingled. Under simulated microgravity, the structural types are conserved, but the nucleolar size is smaller, fibrillar centers are always fewer and larger and the proportion of GC is lower than in the 1 *g* control (**e**–**h**). Bars indicate 1 μm microgravity were significantly smaller than in the 1 *g* control. Cajal bodies were found near the nucleolus in the G2 phase (Fig. 3g, h).

When using synchronic cultures and phase-specific cell cycle subpopulations, the characterization of the cell cycle phases in 1 *g* control conditions confirmed that G2 is the most active phase of ribosome production, and consequently of cell growth (González-Camacho and Medina 2006; Ginisty et al. 1999; Medina et al. 2000), and that simulated microgravity produces a substantial depletion effect on the nucleolar activity, which becomes apparent by changes in the nucleolar structure in all phases, but especially during the G2 phase. The evidence of this reduction was also supported by the data obtained in G2/M phase from nucleolin and fibrillarin, which appeared downregulated in their gene expression and showed lower protein amounts in simulated microgravity. On the contrary, neither S phase nor G1 phase subpopulations showed clear variations in these parameters of ribosome biogenesis, even though quantification of the different nucleolar morphofunctional models in these phases showed an increase in the number of inactive nucleoli under simulated microgravity (Kamal et al. 2019a).

Collectively, all these results show that the cell cycle, ribosome biogenesis, and the nucleolus are sensitive cellular targets of the environmental gravity alteration occurring in spaceflight, particularly detected in meristematic cells. Changes in the auxin transport and distribution can be identified at the origin of these cellular alterations, whose consequences may seriously affect the growth and development of the entire plant.

4 Mechanisms of Adaptation to Spaceflight

It is clear that the spaceflight environment, devoid of the gravitational cue, produces a serious alteration of the biological processes and mechanisms sustaining the life of plants. Gravity is responsible for mechanical signals generated in different organs of the plant due to their weight, and for the production of all-pervasive specific directional cues. In fact, these signals and cues are major drivers of the normal growth and development of plants on Earth. In addition, essential physical processes greatly influencing the mechanisms of biochemical reactions, e.g. gas and liquid flow, are conditioned by the gravity force. As a result, water and gas movements are altered in microgravity, which may cause disturbances in the concentrations of key substances in metabolically active tissues (Porterfield 2002).

This unequivocally confronts plants in space with a suite of environmental conditions with which they are not accustomed to live on Earth, whose consequence is that the spaceflight conditions (microgravity, radiation, confinement, and other factors) are a cause of stress for terrestrial plants. In principle, plants have an enormous plasticity and are capable of surviving and adapting to a great variety of environmental conditions. However, the gravitational stress has some specific features that could result in an adaptive response profoundly different from the responses to terrestrial stresses. Drought, salt, heat, or cold are environmental conditions that have produced a "memory" in plants throughout evolution. Therefore, plants have had the opportunity of developing appropriate responsive mechanisms that remain stored and silent under normal conditions, but emerge and operate when needed. However, gravity is an all-pervasive condition throughout space and time in the evolutionary story of plants, so that no memory of microgravity exists in the plants that currently populate our planet and they must develop totally new adaptive strategies to new environmental signals.

An interesting collateral discussion relative to gravitational stress is to consider whether the real stress-generating condition for plants is the lack of gravity, or the cause of the stress is actually the very existence of the gravity vector. To briefly comment this consideration, we should first realize that, from an evolutionary perspective, the conquest of mainland by aquatic plants around 470 million years ago, when a multicellular species of green algae left the ocean, was a fundamental milestone in the history of life. It indeed made possible a burst of biological diversity, by dramatically increasing the oxygen atmospheric levels and providing a potential source of food which allowed the appearance of new organisms and helped their diversification and expansion across the terrestrial world. Gravity played a chief role in this process. For succeeding in this decisive step, land plants (embryophytes) indeed had to overcome a new environmental stress, which was mostly caused by the existence of the gravity vector, and trigger an adaptive response. They had to develop a mechanism of graviresistance, leading to the built of a rigid body capable of withstanding the force of gravity, as well as the systems necessary to transport nutrients and water against the pull of gravity (Plackett and Coates 2016; Hoson and Soga 2003). No doubt exists that, at that time, the gravity vector was a profound stress condition for plants. Nevertheless, the story of evolution continued with the successful adaptation of plants and the result is that gravity is a fundamental driver of plant growth and development as we know them today, and its removal certainly causes important alterations and triggers the establishment of new adaptation mechanisms.

4.1 Transcriptomic Changes and Adaptation

In recent years, we have experienced an increasing availability of -omic methods, more and more robust, for determining global changes in gene expression at the level of either gene transcription (transcriptomics), or protein mapping in quantity and quality (proteomics), or even detection of epigenetic changes (epigenomics), among others. This has made possible a substantial number of studies dealing with these kinds of changes undergone by plants when they grow in a microgravity environment, either real (spaceflight) or simulated. Transcriptomic studies, using microarray or, more recently, microsequencing (RNAseq) techniques are, by far, the most frequently performed.

Transcriptomic studies have produced a great deal of information on the plant responses to microgravity and spaceflight environment. The processing and interpretation of this information is, however, an arduous task, for different reasons. Spaceflight experiments are much less frequent and are subjected to much more constraints than experiments performed in ground laboratories, and this affects the reproducibility of the experiments and the statistical treatment of data. Furthermore, genotypes, growth conditions, hardware used and developmental periods of experimental samples show a great dispersion among different experiments, seriously compromising the harmonization of data. An important effort of data sharing and harmonizing has been undertaken in the project called GeneLab, under the leadership of NASA and the participation of laboratories from different countries. The project is organized into different analysis working groups (AWGs), one of them specifically devoted to plants (Barker et al. 2020; Ray et al. 2018).

Regarding interpretation of data, some studies emphasize the detection of alterations induced in the space environment, with the objective of accurately defining the gravitational stress (Choi et al. 2019; Johnson et al. 2017), whereas in other cases it is claimed that the transcriptional changes are actually reflecting the mechanism of physiological adaptation of plants to spaceflight, since they have been obtained from viable samples (Paul et al. 2012b, 2017). The fact that plants eventually adapt to survive in space is becoming undisputed, since apparently normal adult plants and flowers can be produced in the ISS, and plant full life cycle (seed-to-seed) has been shown to occur in space (Massa et al. 2013; Musgrave et al. 2000; Link et al. 2014). This is the final and successful plant response to the serious alterations in some processes, essential to plant development, that are induced by the microgravity environment, as described in the preceding sections of this chapter. However, most probably, the adaptive response is more complex than a mere change in the pattern of gene expression occurring as soon as the samples experience the new environmental conditions, as it can be detected in transcriptomic studies performed on young seedlings grown for a few days in spaceflight. It is conceivable that plants could trigger an early and primary acclimation response to the environmental change from Earth to space, to overcome the early alterations that could then be extended throughout successive developmental stages. Further, this acclimation would be followed by later adaptive responses involving more stable genetic and epigenetic changes that would be transmitted to the offspring, allowing the survival of plants in the space environment throughout successive generations. The investigation of these complex acclimation and adaptation processes and mechanisms has not yet been undertaken in depth, and it is one of the most important and decisive challenges of space plant biology, now and for the coming years.

The problem is that, most frequently, analyses were done at a single point of the plant development, preferentially on young seedlings, whereas we lack sequential studies on the transcriptional response to microgravity in which the pattern of gene expression of, e.g., young seedlings and mature plants, is compared. Even, these comparative studies should be extended to consecutive generations. In the very few sequential studies performed, the increased duration of the exposure to simulated microgravity resulted in some attenuation of the alterations produced in the root meristem in young seedlings (Boucheron-Dubuisson et al. 2016). Certainly, only cellular methods were used in this work and the analysis was restricted to early developmental stages.

With all these limitations and uncertainties, it is true that the number of spaceflight experiments performed on plants and analyzed with transcriptomic tools is overcoming a certain threshold, allowing us to get some conclusions that can be considered robust and significant. Some genes or groups of genes have emerged from these studies as representing a core set of functions appearing affected by spaceflight environment. The most prominent constituents of this core set are the oxidative stress pathways, involving the production of reactive oxygen species (ROS) (Choi et al. 2019; Kruse et al. 2020; Correll et al. 2013), the system of heat shock response genes, producing heat shock proteins (HSP), which are molecular chaperones acting to protect and refold proteins in response to cellular damage (Choi et al. 2019; Johnson et al. 2017; Zupanska et al. 2013), and the cell wall remodeling system (Kruse et al. 2020; Johnson et al. 2017; Correll et al. 2013; Kwon et al. 2015). In none of these transcriptomic studies, specific genes for the response of plants to microgravity, or to the spaceflight environment, have been identified, although it is noteworthy that the "unknown biological processes" gene ontology group is a major group enriched in differentially expressed genes (DEGs) after incubation of a plant cell culture in simulated microgravity (Kamal et al. 2019b).

Different genotypes, mostly from the model species *Arabidopsis thaliana,* and even different ecotypes of this species have been analyzed in their response to spaceflight. No phenotypical differences were appreciated between them after growing in the space environment, but the transcriptomic results showed highly significant differences in the number and the identity of genes showing altered regulation in comparison with the respective ground controls. This would mean that each genotype, either mutant or ecotype, used a different strategy – a different set of genes – to physiologically adapt to the environmental change (Paul et al. 2017; Johnson et al. 2017). It is very difficult to discriminate which of the found changes are actually adaptive and which are circumstantial, unnecessary for adaptation and therefore dispensable. The aforementioned definition of an affected "core set of functions," though still incomplete, may help in this discrimination, but the specificities of each genotype, organ (root or aerial part) and even each cell type, in the adaptive response, should be taken into account, thus complicating the task.

A further step in understanding the transcriptional adaptive response of plants to spaceflight is the use of mutational analysis for the identification of key elements required for the adaptation. A candidate gene to play a role in the process is the *Altered response to gravity 1 (Arg1),* which is known to participate in the plant gravity responses on Earth through the relocation of auxin efflux carrier proteins PIN2 and PIN3 upon root gravistimulation and the establishment of the auxin lateral gradient in the root (Blancaflor 2013). An experiment in ISS using the wild-type and an ARG1 KO line allowed the identification of DEGs between the two genotypes and the two environments, spaceflight and ground. The data indicate that a high proportion of genes involved in the adaptation of the wild-type to spaceflight are *Arg1*-dependent. The role of this gene in the adaptation mechanism appears to be related to the endomembrane system and cell wall remodeling (Zupanska et al.

2017). Another suitable subject of mutational analysis of the adaptive mechanisms is the network of heat shock factors (HSFs), a member of the core set of genes involved in the plant response to spaceflight. HSFs are transcription factors that regulate the expression of heat shock proteins (HSPs), evolutionarily conserved, that play major roles in the general mechanisms of stress response of almost all organisms. Since cellular stress is characterized by the accumulation of denatured proteins, HSPs are molecular chaperones that control a correct protein folding (Guo et al. 2016). Among HSFs, the *HsfA2* gene was the highest upregulated gene in an *Arabidopsis* cell culture grown for 12 days in space (Zupanska et al. 2013). Therefore, in a further experiment in ISS, cell lines from wild-type and HSFA2 KO were grown and analyzed for their gene expression profiles in search for the reaction to the space environment of a cell line deprived of a putative basic regulator of their response to environmental stresses. The analysis of the space flown samples of the HSFA2 KO line showed that the number of DEGs under spaceflight conditions, compared to the ground control, was much higher than in the wild-type. Plants deprived of this factor appeared affected by endoplasmic reticulum stress, they had to trigger the unfolded protein response (UPR) pathway as a consequence of protein misfolding events, and, consequently, their adaptation and survival in space required a more intense gene reprogramming, thus confirming the key role of HSFs in these processes. This role is played through regulation of cell wall remodeling, plasma membrane signaling and starch production, which appeared as the functional groups with a higher proportion of DEGs exhibiting the most intense changes (Zupanska et al. 2019).

The third and last example of mutational analysis to be mentioned here concerns the use of two lines, respectively, deficient in two genes known to play a role in root skewing, a phenomenon of deviation of the root directional growth with respect to the gravity vector which has been described and discussed in a precedent section of this chapter. The primary functions of the two genes assayed, namely *Spiral1 (Spr1)* and *Sku5,* are, respectively, the regulation of cortical microtubule dynamics and a copper oxidase activity, anchored to glycosylphosphatidylinositol, acting at the interphase between plasma membrane and cell wall. Both SPR1 and SKU5 proteins are associated with cell wall remodeling, one of the processes commonly identified to take part in the plant response to spaceflight conditions. Furthermore, their skewing pathways are different (see Califar et al. 2020 and the references therein). After growing in space for either 4 days or 8 days, the two mutants showed great differences in their transcriptomic response: *spr1* mutant showed fewer DEGs than the Col-0 wild-type, whereas the number of DEGs in the *sku5* mutant was much higher than its corresponding WS wild-type. The interpretation emphasizes the existence of genotypes potentially better prepared to live in the microgravity environment, thus showing an easier adaptation to spaceflight conditions, as indicated by the number of DEGs involved in the response. If this reasoning is correct, the loss of function of the SPIRAL1 protein positively impacts adaptation, whereas, on the contrary, SKU5 protein plays a role in physiological adaptation and the loss of function of this protein initiates a complex and extensive adaptive response involving the participation of mechanisms of response to deep stresses, such as ABA signaling and pathways affecting membrane stabilization and remodeling (Califar et al. 2020). Interestingly, the number of DEGs in *sku5* is dramatically reduced in 8-day-old seedlings with respect to younger 4-day-old ones. As previously indicated, this sequential study could help to differentiate between the early primary response, mostly giving account of the stress suffered by plants, and the later response, which includes genes involved in the adaptive process.

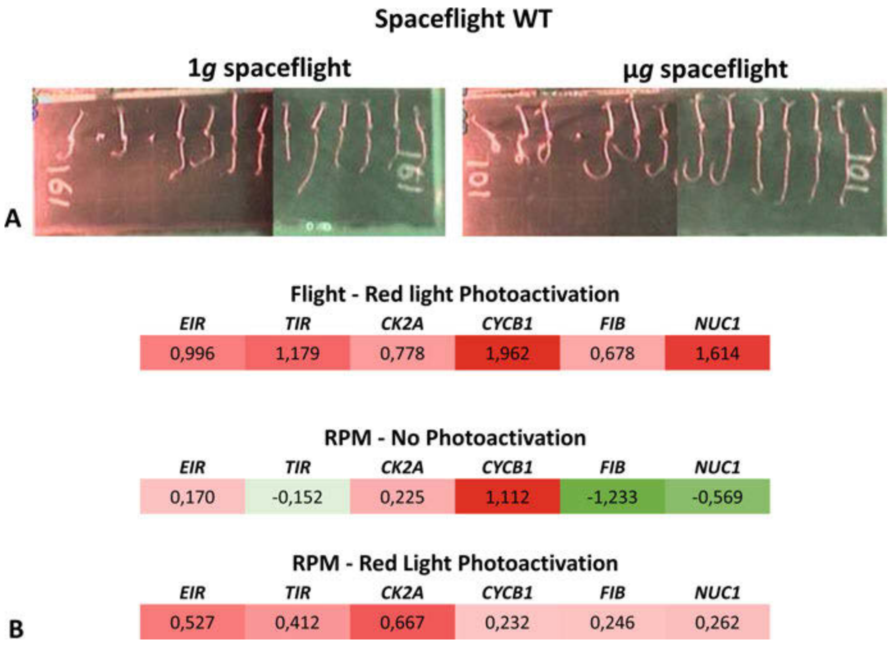
Proteomic studies, intended to establish quantitative and qualitative differences in the protein map of samples grown in microgravity with respect to 1 *g* ground controls, are fewer than transcriptomic analyses. Membrane proteins associated to stress responses (Mazars et al. 2014) and cell wall remodeling factors (Ferl et al. 2015) have been identified in these studies as the most affected functional groups. A recent study combining RNA-seq analysis (transcriptomics) and protein mass spectrometry (proteomics) provided simultaneous data on protein (peptide) abundance and transcript differential expression, as well as of changes in post-translational modifications of etiolated seedlings grown for 3 days on board of the ISS. Soluble and membrane-bound proteins were analyzed separately (Kruse et al. 2020). The study evidenced that gene expression alone is not enough to draw the full picture of the changes induced by space environment. Post-transcriptional regulatory alterations, especially phosphorylation, constitute a highly significant response, which is not revealed by the mere analysis of gene expression. From a functional perspective, cell wall synthesis and remodeling, microtubule dynamics and its interaction with redox homeostasis, and plastid functions appear as the categories most affected by the spaceflight environment (Kruse et al. 2020).

4.2 The Role of Light in Promoting Adaptation

With the objective of enabling a successful culture of plants beyond Earth, the optimization of plant growth in the microgravity environment of spaceflight, as well as in any other condition of reduced gravity could greatly benefit from the substitution of gravity by another external cue, which could play the same or a similar role in driving plant growth and development as gravity does on Earth. Light is a good candidate to be one of these cues, since it is indeed a tropic stimulus. Phototropism complements gravitropism under normal ground conditions with the objective of optimizing the efficiency of the capture of nutrients. In addition, illumination, especially by red light, is sensed and mediated by phytochromes to produce changes in the regulation of auxin responsive genes and many growth coordinators (Vandenbrink et al. 2014). A specific effect of red light in the activation of cell proliferation and ribosome biogenesis had been previously reported (Reichler et al. 2001). Interestingly, enhancement of light signaling to compensate for the absence of gravity was found to be a spontaneous response of plants to the lack of gravitropic stimuli in spaceflight, as shown by the upregulation of genes associated to plastid functioning, some of them closely related to photosynthesis (Kruse et al. 2020). This response took place even in etiolated seedlings, in the absence of an effective light signal. The culture of seedlings in simulated microgravity under a photoperiod regime was capable of reverting many of the alterations found on etiolated seedlings incubated in the same facility for microgravity simulation (Manzano et al. 2020b).

In this context, the series of experiments termed the Seedling Growth (SG) Project was conducted in the ISS. Among other objectives, the project intended to investigate to what extent light can act as a signal capable of counteracting the effects caused by the lack of gravity and to determine the combined influence of light and gravity on plant development by paying special attention to the effect of these cues on the root meristem. The project was the result of the cooperation of NASA and ESA, using a European incubator (European Modular Cultivation System, EMCS) (Brinckmann 2005) combined with an American culture chamber for incubation of seeds and growth of seedlings ("Tropi" cassettes) (Kiss et al. 2009). Different collections of mutants of *Arabidopsis thaliana,* affecting the phytochromes, nucleolar proteins and auxin responsive genes were used. Seeds germinated in flight and grew for 6 days under different regimes of illumination and gravity (Fig. 4). In addition to microgravity existing in space, seedlings were subjected to different levels of gravity between 0 *g* and 1 *g*, including the Moon and Mars gravity levels, which were produced by a centrifuge installed in the incubator.

The experiments have identified new phototropic responses to blue light in space, which complement former findings obtained in the previous Tropi I and II experiments (Vandenbrink et al. 2016). Actually, blue light is a known source of phototropic stimulus in the Earth (Briggs 2014). In samples grown in space under phototropic stimulation with blue light and under different gravity levels obtained with a centrifuge installed in the EMCS facility, a global transcriptomic study provided a very clear differential transcriptional response to each gravity level from microgravity to 1 *g*. In the case of the microgravity exposed plants, functions associated with light sensing and response, such as photosynthesis and related factors, appeared downregulated with respect to 1 *g* controls, suggesting that the growth is not following the phototropic environmental cue in the absence of the gravitropic one. This would mean that gravity responses have an influence on plant development under exposure to directional blue light (Vandenbrink et al. 2019). A similar analysis performed under different levels of gravity, including those corresponding to the Moon and Mars, showed that the effects induced by microgravity were gradually removed by increasing *g*-load, and that different functions appeared affected at different *g*-levels. A strong general abiotic stress response was detected at levels lower than the Moon gravity (lower than 0.1 *g*), probably due to the confluence of different altered stimuli just at the detection threshold of photo- and gravi-sensing mechanisms, which could originate conflicting responses. At higher *g*-levels, the alteration became progressively weaker; membrane- and cell wall-related genes were the most significant DEGs at the Moon g-level, and similar gene ontologies were observed, but were statistically less relevant at increased *g*-levels, such as that of Mars (0.38 *g*). This allowed a discrimination between the differential contribution of the statolith-based gravitropism and other responses based on cell tensegrity that might require a higher *g*-threshold to operate. In general, blue light



Flight - Red light Photoactivation

EIR	*TIR*	*CK2A*	*CYCB1*	*FIB*	*NUC1*
0,996	1,179	0,778	1,962	0,678	1,614

RPM - No Photoactivation

EIR	*TIR*	*CK2A*	*CYCB1*	*FIB*	*NUC1*
0,170	-0,152	0,225	1,112	-1,233	-0,569

RPM - Red Light Photoactivation

EIR	*TIR*	*CK2A*	*CYCB1*	*FIB*	*NUC1*
0,527	0,412	0,667	0,232	0,246	0,262

Fig. 4 (**a**) Images of *Arabidopsis thaliana* seedlings, wild-type Ler ecotype, grown for 6 days in the International Space Station (ISS) in the NASA-ESA experiment "Seedling Growth." Seedlings were grown for 4 days under continuous white light and 1 *g*, followed by two days under lateral red light photoactivation (light source at the left side) and either microgravity (μ*g*) or 1 *g*. (**b**) Transcriptomic study, by qPCR analysis from root RNA extracted from samples that were frozen on-board and recovered from the ISS, and from a successive experiment performed on ground, in simulated microgravity, using a Random Positioning Machine (RPM) (Valbuena et al. 2018). "No photoactivation" means that seedlings were in darkness for the two last days of growth. Six genes from three sets were selected as markers of different functions: *EIR* and *TIR* are genes involved in auxin polar transport and perception; Casein Kinase 2 *(CK2A)* and Cyclin B *(CYCB1)* are involved in cell cycle regulation/cell proliferation rate; Fibrillarin *(FIB)*, and Nucleolin 1 *(NUC1)* are involved in the regulation of ribosome biogenesis, indicative of cell growth. Differential expression of these genes was measured in comparison with their respective 1 *g* control (log2Ratio), either in flight or in ground, and expressed with a color code in which red represents upregulation and green downregulation. With red light photoactivation, genes responsible for cell proliferation and cell growth are all upregulated, whereas in darkness, cell proliferation is upregulated and cell growth downregulated. This means that red light photoactivation was capable of restoring meristematic competence, which appeared disrupted in simulated microgravity and darkness, the same as it had been previously found in a former experiment in ISS (Matía et al. 2010). Adapted from Valbuena et al. (2018)

phototropism was found to be capable of reducing the gravitational stress response on orbit (Herranz et al. 2019).

Regarding the effect of red light, a positive effect of photoactivation with this wavelength in counteracting the stress caused by spaceflight on cell growth and proliferation in the root meristem has been found in wild-type samples (Valbuena et al. 2018) (Fig. 4). Unilateral illumination with red light during the last 2 days of culture, after 4 days of growth under white light photoperiod in the ISS, was capable of reverting (totally or partially) the alterations caused by microgravity on the root meristem. This included re-establishing meristematic competence and auxin transport. The analysis was performed by selecting marker genes for these processes and analyzing by qPCR their differential expression under different conditions of gravity and light. A parallel study on ground using simulated microgravity (RPM) confirmed the spaceflight data (Valbuena et al. 2018) (Fig. 4). In addition, the localization of nuclear proteins and auxin distribution were analyzed by the confocal and electron microscopy. It should be noted that the *in situ* results have been obtained with the plant tissue fixed in aldehyde fixatives on board of the ISS. This experiment has provided a substantial advance, firstly in the technology development with the design of a device to chemically fix samples – Fixbox (Manzano et al. 2020a), and secondly in the understanding of the *in situ* plant response to the microgravity conditions. An auxin accumulation in the root tip was observed, indicating some alterations in the auxin polar transport, confirming previous experiments on simulated microgravity (Herranz et al. 2014).

The use of nucleolin mutants could be integrated in the knowledge of the plant response to spaceflight environment by means of mutational analysis discussed in the preceding section. As previously discussed in this paper, ribosome biogenesis is a cellular process that plays an essential role in plant growth and development, it is a complex stress-sensitive process and it has been found to be seriously altered under conditions of spaceflight and microgravity, both real and simulated. In the process of ribosome biogenesis, nucleolin is a multifunctional nucleolar protein that occupies a central position in its regulation, acting at different steps. Furthermore, two specific features of nucleolin in plants, and particularly in *Arabidopsis thaliana,* provide additional advantages to the use of nucleolin mutants in the SG series of spaceflight experiments. Firstly, plant nucleolin gene expression is known to be upregulated by red light on Earth (Reichler et al. 2001). Secondly, from the two nucleolin genes of *A. thaliana,* one of them, *Nuc2,* is known to participate in the mechanisms of adaptive responses to different stresses (Durut et al. 2019). A global transcriptomic study performed on ground using WT and both *nuc1* and *nuc2* mutant lines, comparing red light photoactivation conditions for 2 days *versus* darkness, has revealed that the capability of the *Nuc2* gene of replacing the functions of *Nuc1* in ribosome biogenesis and cell cycle, when this gene is not present, is enhanced by red light. In addition, photoactivation induces in the *nuc2* mutant an increase in DEGs belonging to functional groups associated to stress response. This means that the *Nuc2* gene may counteract the environmental stress produced by darkness in *nuc1* plants and *nuc2* plants cannot develop a full response to red light (Manzano et al. 2020c).

A full-genome global transcriptomic analysis of red-light-photostimulated plants *versus* plants grown in darkness, in the SG spaceflight experiment, showed that, in photostimulated plants of WT, *nuc1* and *nuc2*, a lower number of genes whose expression is dysregulated by microgravity were obtained, in comparison with plants grown in darkness during the same period. Specifically, the *nuc2* mutant adapted to the microgravity environment by changing the expression of a smaller number of genes than the wild type. Therefore, a mutant line with this attenuated response may constitute an advantage to be taken into account when selecting the most productive plant varieties for Life Support Systems. The study at the cellular level of the cell cycle and ribosome biogenesis in root meristematic cells resulted in values closer to the 1 *g* control in those samples stimulated with red light.

5 Future Prospects

Most of the plant biology research on ISS has been performed on model plants and there is a lack of fundamental understanding of cultivation and adaptation of crop plants to the space conditions. Nowadays, there is a higher demand for fundamental research that goes beyond the demonstration of plants ability to adapt to the reduced gravity. Bridging the gaps in the knowledge of the acclimation/adaptation mechanisms and effects on the plant development in the space environment through the whole life cycle of a plant, including crop plants, is currently only possible using the ISS research platform and this knowledge is required to ensure reliable and predictable food supplies in future human space exploration. To understand the adaptive processes, long-term responses have to be investigated through sequential studies of plants after different times of exposure to altered gravity and at different phases of the plant development.

Some of the pending basic questions whose answer is necessary to understand how plants acclimate and then adapt to the space conditions refer to our still incomplete knowledge on the signal mechanisms, cell proliferation, cell cycle, cell differentiation, plant tissue, organogenesis and whole plant functioning after the exposure to the spaceflight environment over time during different developmental phases. Together with these topics, other fundamentals aspects of plant biology have been very little or no investigated, such as the maintenance of the plant posture in space and the contribution of proprioception, in comparison with light sensing, to the plant posture in space, the effects on the water transport system and photosynthetic organs and the photosynthetic machinery, the nutrient uptake and transport of water and solutes, the reproductive biology (*e.g.,* sporogenesis, gametogenesis, fertilization, embryogenesis) and the effects of chronic radiation exposure on the plant response to altered gravity.

Different -omics research experiments will play an increasingly chief role in approaching these objectives, but establishing cross-comparisons between the transcriptomic data from different plant spaceflight experiments is now mandatory. New consortia are appearing at the European and International levels (Madrigal et al. 2020; Rutter et al. 2020) to support GeneLab database curation of the data, providing more insight and common criteria for the exploitation and comparison of the datasets. There is also a need for the spaceflight community to be aware of the constraints of the spaceflight research, including a particular care in the preparation and analysis of ground-based reference experiments and controls (Manzano et al. 2020c). Recent reviews present to the scientific community the current opportunities for space -omics research and discuss how to give to space biology research the opportunity to meet good practice standards similar to those of other plant biology studies, in the attempt to avoid the criticisms that are used from researchers outside of the research field (Overbey et al. 2020; Afshinnekoo et al. 2020).

From an applied point of view, a better understanding of all these topics will pave the road toward bioregenerative life support systems, allowing plants to be used as food for crew and as part of regenerative processes in future missions deeper into space. Additionally, understanding how plants are able to grow in space will have strong synergies with food security and circular economy on Earth.

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Terrestrialization: The Conquest of Dry Land by Plants

Ulrich Lüttge

Contents

Abstract Life originated in water. Then there was terrestrialization, the conquest of dry land. This was a necessity because of the drive for occupation of any possible space being an intrinsic property of life. Plants had to go first before animals and further progress in terrestrialization within coevolution. The move of life from water to dry land may have occurred in a number of separate steps. It is probable that there were two waves of terrestrialization. Looking at extant processes of the occupation of adverse surfaces a first wave likely was the formation of biofilms and biological

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soil crusts on land by cyanobacteria and green algae. In the soil crusts, the more advanced thallophytes of bryophytes and lichens having evolved on land joined in. Consequently, the second wave was that of the vascular plants. The early, but now extinct vascular plants were the Psilophytopsida. They lived in flat water bodies near the shore of lakes or in wet depressions. Some of them were still aquatic, some were amphibious, and others were terrestrial. They could have reached the land when water bodies temporarily had fallen dry and also by wind-driven flow of spores from shoots sticking out of the water. This terrestrialization began in the Upper Silurian/Lower Devonian, 420–410 × 10^6 years ago. Vascular plants on dry land simultaneously live in two contrasting environments, the pedosphere and the atmosphere. In the pedosphere, they need to procure minerals from the bedrocks. Soils had to be formed originating through plant activity and decay. Bedrock exploitation and soil development are supported by the symbiosis with fungi spreading their hyphae in the ground. This plant-fungal mutualism was present and obviously already essential in the early psilophytes. This is the reason why terrestrial plants are sessile and need to be stationary. Photosynthetically active green plants had to go on land first because of their basic services for the evolving terrestrial compartment of the biosphere. First, as autotrophic organisms capable of reducing and assimilating CO_2, NO_3^-, and SO_4^{2-}, green plants are the primary producers of biomass feeding the food webs of life. Second, through the process of photosynthesis plants evolve oxygen, O_2, and the evolution of an atmosphere with increasing levels of O_2 was essential for the respiration of animals. Third, the evolvement of O_2 allowed formation of a stratospheric ozone-shield, O_3, protecting all life on land from destructive solar ultraviolet radiation. Unicellular algae in the water already show preadaptations to stress on land, in particular in view of desiccation tolerance. Lignin is essential for biomechanics of plants on land, and there are already lignin-type compounds in algae. The bipolar gestalt, which is so characteristic of the life of land plants in pedosphere and atmosphere, had already evolved in algae in the water. Terrestrialization by plants was a necessity in conquering new space and gaining competitive advantages. Therefore, evolution of life on land appears as a compelling consequence of preadaptations already acquired by early aquatic life forms.

1 Introduction

Life originated and evolved in water. Internally all organisms by volume and weight consist of water to high percentages, often more than 90%. Externally water appears as a perfect medium. It contains minerals and other solutes and avoids drought. By contrast, the land outside the water early on our planet was a forbidding environment. So, why was there the conquest of dry land by life? Terrestrialization poses many more or less difficult questions. Some of them can be addressed by observations of extant life. Others can only be approached by speculation and philosophy.

Was terrestrialization an evolutionary necessity? There are questions of how and why it happened.

For the question of WHY there was terrestrialization and why it may even have been necessary, one has to be aware that space is a fundamental resource (Grams and Lüttge 2010). As populations grow and expand space increasingly gets limiting. Fierce competition arises between different populations and between individuals within populations. Hence, the conquest of new open space becomes essential. It appears as a basic property of life to occupy any space accessible.

How did terrestrialization proceed? Why had green organisms and plants to move to the dry land first, followed by animals? Through coevolution both together then shaped the whole appearance of Earth. Terrestrialization was an enormous evolutionary success. The most advanced forms of plant and animal life evolved on land. Life is conditioning the standing of our planet as we know it (Lüttge 2016; Lovelock 2019).

Regarding the question of HOW, we can address the major environmental challenges for early life on land. On such grounds, we may ask if there are traits in algae which would make them fit for moving from life in the water to dry land. Then we may consider extant algae, so-called aerial algae, which have achieved that, along with other more advanced terrestrial thallophytes. Presumably, the conquest of land occurred more than once in evolution. We can also study the fossil records of early vascular plants growing in shallow waters from where they advanced to the dry land. Why do dry land conditions require plants to become sessile and stationary as opposed to freely moving animals?

Terrestrialization was directed evolution from water to land. Given that preadaptations of algae and early vascular plants promoted traits supportive of life on land, we may ask if there were genes on stock. Did gene duplications with changes of gene functions, horizontal gene transfer, and gene gains produce a molecular toolkit, from which preadaptations originated as devices assembled in a toolbox for further adaptive developments of terrestrialization? Perhaps appearing as necessities in evolution and allowing directed selection beyond chance? Recognizing features of directedness leads to a more philosophical outlook revisiting the concept of teleology.

2 Environmental Challenges of Life on Dry Land

The major problems for plants on dry land are

- water relations,
- nutrition,
- mechanical support,
- high irradiance and UV,
- temperature extremes,
- propagation, distribution, and reproduction,
- occupation of space.

Table 1 Traits in extant algae in the Division Chlorophyta and the Sub-Division Streptophytina of the Sub-Realm Chlorobionta towards fitness on dry land

Division Chlorophyta	
Class Ulvophyceae	Bipolar filaments
Class Chlorophyceae	
Order Chlamydomonadales	Multicellular colonies
Order Chlorococcales	Desiccation tolerant land algae
Order Chaetophorales	Bipolar thalli with soil sole
Class Trebouxiophyceae	Desiccation tolerant land algae
Class Trentepohliophyceae	Desiccation tolerant land algae
Division Streptophyta – Sub-division Streptophytina	
Class Zygnematophyceae	Desiccation tolerance genes
Class Coleochaetophyceae	Bipolar thalli with soles, lignin-like compounds
Class Charophyceae	Complex branched bipolar thalli with rhizoids

Evolution of the complex plant bodies of vascular plants inhabiting land has produced well-adapted structures and functions in response to such challenges. Cooperation of roots and shoots (Sect. 6) by long distance transport of water (Sect. 5) and regulation of transpiration by stomata settle water relations and mineral nutrition. In aquatic environments, mechanical support is given by buoyancy even of very large plant bodies as encountered among brown algae, such as *Macrocystis pyrifera*, which may reach sizes of more than 100 m. On land, biomechanics of tall plants required the chemical evolution of lignin, which like in reinforced concrete encrusts the cellulose cell walls (Sect. 3.4). In water, gametes for sexual reproduction and diaspores for distribution can drift and swim. On land, special mechanisms evolved based on wind and mutualism with animals.

In exploring by evolutionary/developmental biology (EvoDevo) the early steps of evolution and development towards the occupation of land, preadaptive algal traits deserve attention (Sect. 3). In extant algae, we observe traits which will provide fitness on dry land. These are particularly desiccation tolerance, loss of mobility and formation of bipolar thallus bodies, and the production of lignin-like compounds. They will be discussed in the following Sects. 3.1–3.4 and are summarized in Table 1 (see also Fig. 1). We envisage that most likely the conquest of land by green plants occurred not just once but by several, evolutionarily separate waves. From what we learn by looking at algal traits for fitness on dry land, we can deduce two major pathways of the acquisition of terrestrial space. One is the coverage of terrestrial surfaces by mainly unicellular desiccation-tolerant cyanobacteria and algae (Sect. 4). The other one led to the establishment of bipolar early vascular plants (Sect. 5).

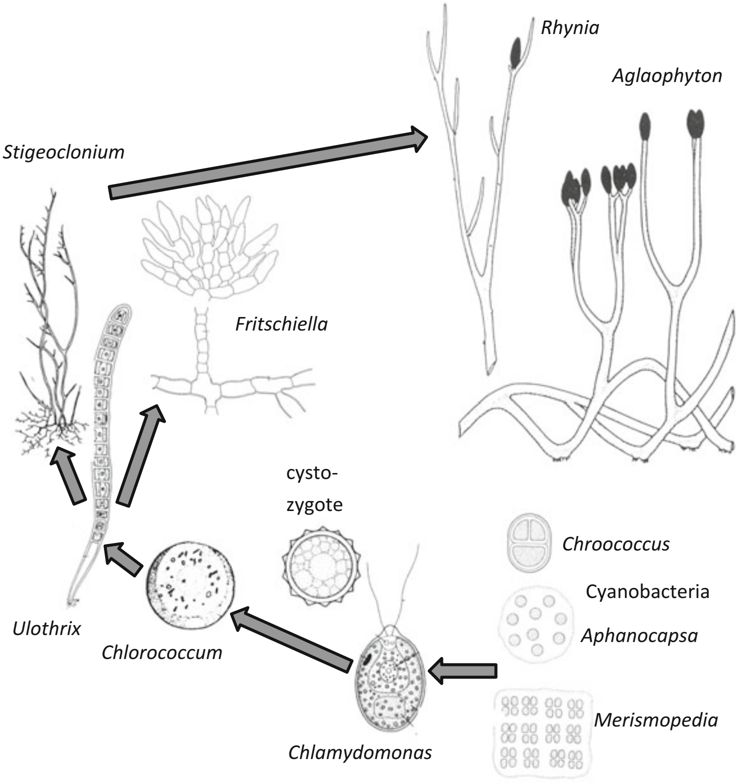

Fig. 1 Putative evolutionary trends of forms of green organisms on the way from life in the water to life on the dry land as described in the text. Note that the various forms are reproduced at different scales. (All pictures are extracted from figures in Bresinsky et al. (2008), namely from Figs. 10–13, 10–114, 10–108, 10–117, 10–119, and 10–152)

3 Algal Traits for Fitness on Dry Land

3.1 Molecular-Genetic Bases

All Higher Plants on land are Streptophyta, which have originated during evolution from streptophyte algae (Sect. 3.3). Molecular genome studies show that in the earliest-diverging clade of streptophyte algae there were genes and metabolic pathways supporting dehydration tolerance and UV-protection, and hence paving the path to land (Wang et al. 2019; DeVries and Rensing 2020; Tena 2020). These studies include the flagellate *Mesostigma viride* living in the benthos of small shallow ponds and the subaerial-terrestrial *Chlorokybus atmophyticus* as a partner in biological soil crusts (Wang et al. 2019).

3.2 Desiccation Tolerance

Homoiohydrous plants must maintain their water relations under close and steady control. In contrast, there are poikilohydrous plants which can undergo cycles of drying, dormant dry states, and rewetting. If able to overcome periods of almost complete dryness, such plans are termed desiccation tolerant.

3.2.1 Distribution of Desiccation Tolerance Among Green Plants

Desiccation tolerance is a trait supporting life outside water. It is an effective means to overcome the drought stress on dry land. In higher land plants, the vascular plants, however, whole plant desiccation tolerance is rare. A few ferns are desiccation tolerant. Desiccation-tolerant species are lacking among the gymnosperms. There are only about 1,300 desiccation-tolerant species of angiosperms (Porembski 2011). Nevertheless, desiccation tolerance habitually warrants seed viability (Angelovici et al. 2010; Grene et al. 2011). Conversely, there are many cyanobacteria and algae living on land, so-called aerial or atmospheric and also soil algae, which are desiccation tolerant (Sect. 4.1). Even in aquatic unicellular algae stages in life cycles suggest desiccation-tolerance, given that cysts and spores are formed, or cysto-zygotes as in the case of *Chlamydomonas monoica* (Bresinsky et al. 2008, p. 730; Fig. 1). These are dormant states, often surrounded by thick cell walls, withstanding adverse environmental conditions. Among the advanced thallophytes, many bryophytes and lichens are desiccation tolerant (Sect. 4.3).

Desiccation tolerance of prokaryotic cyanobacteria shows that this is a very early trait in evolution (Bartels and Hussain 2011). That it occurs in organisms still restricted to life in water underlines this trait being a preadaptation for the later conquest of dry land. In early diverging algae of the streptophyte family, Zygnemophytaceae genes increasing desiccation resistance were identified (Cheng et al. 2019; Wang et al. 2019). Early in the evolution of land plants, it is in the bryophytes that we find desiccation tolerance expressed very much (Lakatos 2011; Green et al. 2011; Heber and Lüttge 2011).

3.2.2 Desiccation Tolerance Traits in Cyanbobacteria

Cyanobacteria can lose water to almost complete desiccation of 0.1 g H_2O/g DW (Billi and Potts 2002). Such desiccation is the extreme endpoint of moderate drying. An array of cell physiological responses is effective in plants and many of them are already expressed in cyanobacteria (overview and references in Lüttge 2011). The formation of so-called compatible solutes of osmolytes, such as sugars, sugar derivatives, polyols, and betains, stabilizes macromolecules and membranes when cells lose water. Heat shock and water stress proteins are stable for decades of dormant storage in the dry state (Sect. 4.1.1). Effective sun-blocking pigments, such as mycosporine-like amino acids and the indole-alkaloid scytonemin, provide UV-protection (Sect. 4.1.1). There are responses of membrane-lipid composition. Polynucleotide stability and repair are conspicuous.

Desiccation-tolerant cyanobacteria are homoiochlorophyllous, i.e., chlorophyll is maintained during desiccation. Dangerously, chlorophyll remains excitable, however, by light absorption while the excitation energy cannot be dissipated anymore by the metabolic reactions of CO_2 assimilation. As countermeasures, protective mechanisms of harmless energy dissipation controlling photoinhibition and photodestruction have been developed. Recovery of photosynthesis upon rewatering after desiccation depends on the length of the dormant state and can take a few minutes up to several hours. Cyanobacteria are also capable of atmospheric di-nitrogen, N_2, assimilation (Sect. 4.1.1), which recovers after desiccation, i.e., within minutes to hours after dormancy of a few days and, in some cases, within 120–150 h after dormancy of 2 years (Lüttge 2011).

3.3 *Loss of Mobility*

Plants on land are stationary and not freely mobile as in water. Loss of mobility evidently was an important step towards the establishment of plants on land. Evaluating this as a preadaptation for terrestrialization we here consider algae of the Division Chlorophyta and the Sub-Division Streptophytina in the Sub-Realm Chlorobionta (Table 1), because all the extant vascular land plants, i.e., the ferns and fern allies (Sub-Division Pteridophytina) together with the seed plants (Sub-Division Spermatophytina), are in the Division Streptophyta of this Sub-Realm (taxonomy according to Lüttge et al. 2010). Of particular interest are evolutionary trends in the Chlorophyta with the Class Chlorophyceae and its Orders Chlamydomonadales (syn. Volvocales) and Chlorococcales and the Class Ulvophyceae.

The unicellular alga *Chuaria circularis* occurred worldwide 10^9 years ago (Frey and Lösch 2004). The simplest extant eukaryotic algae are unicellular forms of the Chlamydomonadales, e.g., *Chlamydomonas* (Fig. 1), swimming in the water with a pair of flagella. Colonies evolved from vegetative cell divisions, as daughter cells remained attached within a secreted gel, e.g., 4–16 cells in *Gonium*, 4, 8, or 16 cells in *Stephanosphaera,* and 16 cells in *Pandorina*, forming small spheres in the latter case. *Eudorina* and *Pleodorina* are hollow spheres of 32 and 128 cells, respectively. All of these colonies are mobile as their cells keep the flagella. The climax of this evolution is in the genus *Volvox* with 16,000 cells in *Volvox globator*, where the cells at the surface of the hollow sphere are connected by plasmodesmata and a certain degree of specialization is expressed with a front and a rear pole. *Volvox* are truly multicellular organisms (Bresinsky et al. 2008; Lüttge et al. 2010; Layer and Lüttge 2020). Nevertheless, their evolution led to a dead end. No further developments started from *Volvox*, which had also appeared rather late in the geological history of plants, i.e., 200–240 $\times 10^6$ years ago (Frey and Lösch 2004; Herron 2016), at times when there were already highly differentiated vascular land plants. The evolution to precursors of terrestrial Higher Plants went a different way.This evolution was coupled with the loss of flagella and therefore mobility. Some normally mobile forms overcome adverse conditions, such as drying out of waters, by losing the flagella and by forming cysts as endurable dormant states. Some immobile unicellular algae, e.g., *Chlorococcum* (Fig. 1), still form mobile forms after vegetative reproduction for dissemination but then lose the flagella. In the Order Chlorococcales, we also find immobile colonies of *Scenedesmus* and *Pediastrum* and the hollow spheres of *Coelastrum*.

The early evolutionary stages towards advanced immobile plant life can be seen in the Class Ulvophyceae of the Chlorophyta. *Ulothrix* is a genus of filamentous algae in marine and freshwaters. *Ulothrix zonata* (Fig. 1) produces mobile zoospores in vegetative propagation. The zoospores then lose their mobility and begin to divide. Attaining immobility, it is now possible that the daughter cells stay one-dimensionally aligned so that longitudinally growing filaments are formed. Each cell can divide in terms of intercalary growth with the exception of the lower terminal cell which forms a basal unicellular rhizoid. In this way, for the first time, the spherical habit is abandoned and a bipolar longitudinal algal body is created.

Among algae, evolution has sustained and advanced this bipolarity to more perfection in the Order Chaetophorales of the Chlorophyceae and in the Classes Coleochaetophyceae and Charophyceae, which are not in the Division Chlorophyta anymore but in the advanced Sub-Division Streptophytina of the Division Streptophyta within the Chlorobionta. Genera of the Chaetophorales, such as *Stigeoclonium* and *Fritschiella*, have a differentiation of branched filaments on the ground and upright filaments (Fig. 1). *Stigeoclonium* is a freshwater alga, while *Fritschiella* lives on dry land on soil in India and Africa. Its growth habit foreshadows the division of labor between roots and shoots in higher land plants (Bresinsky et al. 2008, pp. 734–735). The freshwater alga *Coleochaete* is filamentous but additionally forms disk-like soles. Characeae represent rather complex branched thalli growing with terminal cells on the main axis and the various branches and being anchored with long rhizoids in the mud of shallow freshwater. Here dense submerged meadows may be formed up to half a meter high.

The evolutionary trends in the chlorophyte algae towards stretched bipolar thalli mark the way to forming stationary Higher Plants on land. Remarkably, also in other Sub-Realms differentiation of the algal bodies has much advanced towards complex thallus-structures of phylloids, cauloids, and rhizoids as encountered in the brown algae (Sub-Realm Heterokontobionta, Class Phaeophyceae) and red algae (Sub-Realm Rhodobionta, Class Rhodophyceae). However, these algal groups have not taken over dry land. All higher land plants belong to the Sub-Realm Chlorobionta. This raises the intriguing question of why that is so. Why did the algae in the other Sub-Realms not make it? Is it because the genetic-molecular toolkit present in the early-divergent Chlorobionta (Sect. 3.1) was missing, although we know that a terrestrial red alga, *Porphyridium*, exists (Sect. 4.1.2)? More comparative genomic analyses including *Porphyridium* may elucidate that issue.

3.4 Lignin

The reinforced-concrete type of cell walls is a prerequisite for the mechanical support of upright growth of plant bodies above the ground on dry land. This depends on lignification. Lignins are polymers of monolignols, i.e., phenyl-propane alcohols, which are released from the living cells, penetrate and imbibe the interfibrillar spaces of the cellulose walls and polymerize there as an encrustation. This encrustation corresponds to the concrete which is reinforced by the cellulose fibers, functionally resembling the steel framework. It is claimed, that the evolutionary invention of lignin with its complex biochemistry and cytology was a decisive step required for the establishment of plants on land.

Searching for preadaptations it is, therefore, exciting to find lignin-type compounds in aqueous algae. The zygote of *Coleochaete* is covered by cellular filaments forming a so-called zygote-fruit which is protected by lignin-type compounds (Bresinsky et al. 2008, p. 742). Regarding the bryophytes, lignin is thought to be an apomorphic trait towards the evolution of land plants (Lüttge et al. 2010). However, there may have been some carry-over from algae regarding lignin-type compounds in the cell walls of the moss-genus *Sphagnum* growing in moist peat bogs (Bresinsky et al. 2008, p. 753).

4 Conquest of Dry Land by Desiccation-Tolerant Thallophytes

Among the extant green terrestrial organisms on Earth, the phylogenetically old basal lineages of the cyanobacteria and the green algae and to some extent also the more advanced thallophytes of the bryophytes and lichens are closest to aquatic origins. Therefore, studying their comportment in using open space on land can tell us much about how the geologically early conquest of dry land by these life forms may have been achieved. However, only a rather rough putative timescale can be deduced from these comparisons (Table 2).

4.1 Aerial Cyanobacteria and Green Algae

Any surface on Earth not occupied by other kinds of vegetation and therefore free of competitors is used by the growth of cyanobacteria and unicellular algae. Such surfaces may be extremely hostile environments with adverse conditions, such as sand in deserts and savannas, bare rocks, and the walls of buildings. This acquisition of open space on dry land by extant cyanobacteria and algae in analogy makes it most likely that similar events occurred in the early terrestrialization of plant life.

Table 2 Rough time scale of possible terrestrialization by various groups of green organisms

Organisms	Comments	Beginning of terrestrialization (millions of years in the past)
Start of first wave of terrestrialization		
Cyanobacteria	Early fossil stromatolites	3,500
Eukaryotic algae	All major algal groups had evolved in the Silurian	440–410
Bryophytes	Origin Silurian – Devonian oldest fossils	360
Lichens	Evolved in the tertiary beginning with the Paleocene	60
Start of second wave of terrestrialization		
Vascular plants	*Cooksonia* oldest fossils of vascular land plants	420–410

4.1.1 Terrestrial Prokaryotic Cyanobacteria

The oldest fossils of living cells are stromatolites. These are layers of cyanobacteria occurring in shallow waters as still formed to-day. Fossil stromatolites are $3{,}500 \times 10^6$ years old with a wide distribution at that time (Byerly et al. 1986). Terrestrial cyanobacteria are pioneers for the establishment of life on bare inorganic support (Lüttge et al. 1995; Lüttge 1997). They form biofilms on the bare sand in savannas and deserts. Very conspicuously with a high species diversity (Büdel et al. 1994) they grow on all exposed rock surfaces in the tropics (Lüttge 2008), but actually globally on Earth, on granite, dolomite limestone and sandstone, and even on anthropogenic substrates such as concrete buildings (Lüttge 1997; Büdel 2011a). Dominant stressors are irradiance, heat, and shortage of water (Lüttge 1997). Where water is running down on rocks in the mountains, or on concrete buildings after rainfall, or during snow-melt cyanobacteria form black strips, the so-called ink strips. They are desiccation tolerant (Sect. 3.2.2). In Antarctica, cyanobacteria become freeze-dried at times (Büdel 2011a). On granite rocks in the tropics, drainage furrows provide microhabitat niches through different drying kinetics after water run-off in the furrow center, on the lateral slopes and on horizontal rock areas outside furrows. The niches are occupied by cyanobacteria with different species communities. In the center, the cyanobacteria are subject to shear forces by the stream of water (Lüttge 1997; Ziegler and Lüttge 1998; Rascher et al. 2003). With their vigorous life under these adverse conditions, the extant sites of terrestrial cyanobacteria may be considered as analogous to early terrestrialization by such prokaryotic organisms. Particularly three properties make cyanobacteria fit for such habitats (Lüttge 2008, 2011):

- they are well adapted to very high solar irradiance and UV and the associated heating up of their substrate,
- they are desiccation tolerant (Sect. 3.2.2), and,
- they can gain nitrogen by fixation of atmospheric N_2.

For resisting solar irradiance cyanobacteria produce sun-blocking pigments such as mycosporine-like amino acids and the indole-alkaloid scytonemin (Garcia-Pichel and Castenholz 1991; Büdel et al. 1997; Büdel 1999). The photosynthetic apparatus is protected from photo-damage by carotenoids (Demmig-Adams et al. 1990; Lakatos et al. 2001). For the control of heat stress heat-shock proteins (Adhikari 2003) and water stress proteins (Scherer and Potts 1989; Hill et al. 1994) are accumulated.

The nitrogen fixing enzyme nitrogenase is sensitive to oxygen. Therefore, N_2 fixation in many cyanobacteria is located in special cells, the heterocytes, which have thick walls limiting O_2 influx from the atmosphere and which lack photosystem II and hence photosynthetic O_2 generation. Besides using the assimilated N_2 for their own growth there is a substantial input of reduced nitrogen to ecosystems by the cyanobacteria, e.g., 3.5, 18, and 130–235 kg ha^{-1} $year^{-1}$ assessed at three different locations in the tropics (see Lüttge 2008).

4.1.2 Terrestrial Eukaryotic Algae

Eukaryotic green algae are much younger than the prokaryotic cyanobacteria, but by 500–400 $\times$ 10^6 years ago in the Silurian all major groups of algae had evolved. The large habitats and the considerable species diversity of terrestrial eukaryotic algae are described by Büdel (2011b).

Lithophilic algae inhabit rock substrates globally, including the Antarctic. The rocks are granite, sandstone, or dolomite limestone. Desiccation tolerance is the major adaptation required. At locations in high mountains and in the Antarctic desiccation is combined with freezing and additionally freezing tolerance is relevant. The algae can be *epilithic* colonizing exposed rock surfaces or *endolithic* expanding a few millimeters into the interior of rocks below the surface. *Hypolithic* algae grow below translucent rocks where besides green algae also diatoms can be found.

Soil algae can amount to up to 100,000 algal cells per gram of soil in the uppermost layer. *Spongiochloris* is thermotolerant (Bresinsky et al. 2008). Green algae per se can also form *soil crusts* on the top of soils by gluing loose soil particles together which reduces erosion by wind and water and fixes sand surfaces in savannas, deserts, and dunes. However, algae are also important parts of biological soil crusts which constitute terrestrial ecosystems of a special kind (Sect. 4.4).

Epiphytic algae in the temperate zones and in the tropics cover tree barks and particularly in the tropics also grow on leaves epiphytically. Prominent genera are in the Chlorophyta *Desmococcus*, *Apatococcus*, *Coccomyxa*, *Trebouxia* (Trebouxiophyceae) and *Trentepohlia*, *Pleurococcus* (Trentepohliophyceae), and there is also the red alga *Porphyridium*. They are all desiccation tolerant. The kinetics and degree of recovery depend on the length of the desiccation state. Different bark types, e.g., of *Fagus sylvatica*, *Acer* sp. and *Malus* sp., constitute different ecophysiological niches for the algae that can be distinguished by their capacity to recover from desiccation after different times in the dry state (Lüttge and Büdel 2010).

Airborne algae are carried by dust storms globally around the planet. Büdel (2011b) lists 58 genera of Chlorophyta as well as more than 30 of cyanobacteria, all being desiccation tolerant. Algae form resting cells of cysts and spores.

4.2 Seaweed Algae in the Intertidal Zone

Marine algae in the tides along rocky coastlines are periodically flooded and dry, respectively. The periodicity implies extreme stresses during low tide, comprising partial desiccation, high-light and UV exposure, freezing depending on latitude, dramatic changes in salinity, and mechanical forces as tides move in and out. Thus, temporarily the algae experience the harsh conditions as prevalent on land surfaces during early Earth history. Therefore, the view was held for some time that the morphologically advanced marine algae or seaweeds from the intertidal zone might have been ancestors of real land plants (Bresinsky et al. 2008; Lüttge et al. 2010).

This view has changed. Although some green algae exist in the intertidal zones, these habitats are dominated by brown and red algae. However, the streptophyte Charophyceae, from which the land plants evolved, live in shallow fresh water bodies. These can seasonally fall dry and then the algae experience conditions of terrestrial life. Thus, it appears most likely that shallow freshwater was the starting point of early terrestrial vascular plants (Sect. 5).

4.3 Bryophytes and Lichens

While the cyanobacteria and algae first conquering the dry land arrived there after evolution in the water, bryophytes, and lichens are primary land plants. The few mosses that live in water are secondary water plants. Having evolved on land bryophytes and lichens are morphologically more differentiated thallophytes than the unicellular or filamentous algae. Fossils of bryophytes, mosses, and liverworts are known from the upper Devonian and Carboniferous (Bresinsky et al. 2008). Mosses are complex multicellular organisms. They have developed cauloid stemlets, phylloids, and rhizoids. Lichens represent complex symbioses between fungal hyphae and cells of cyanobacteria (cyanolichens) and green algae (chlorolichens). Both bryophytes and lichens were among the first groups of more evolved organisms to colonize land at some time before the Silurian when the first vascular plants arrived 420–410 × 10^6 years ago (Green et al. 2011; Lakatos 2011). The bryophytes are rather old and may date back to that time, but the oldest fossils of lichens are from the Tertiary and only less than 60 × 10^6 years old (Table 2).

Mosses and lichens are poikilohydric plants and very many of them are desiccation tolerant (Green et al. 2011; Lakatos 2011). Although many species especially among the mosses prefer moist habitats, extant bryophytes and lichens dominate sites where vascular plants do not grow, including rock surfaces (Lüttge et al. 2008), and epiphytic sites with low environmental water storage and only transient availability of water. Evidently, they must have been fit for early terrestrialization.

Desiccation-tolerant poikilohydrous bryophytes and lichens retain their chlorophyll in the dry state, they are homoiochlorophyllous. This causes the very dangerous situation that light is absorbed by chlorophyll and excitation energy is generated which cannot be dissipated by photochemical work of CO_2 assimilation in the dry state. These plants possess very specific and highly efficient ultrafast mechanisms of energy dissipation, enabling photo-protection of their photosynthetic apparatus in the dry state (Heber and Lüttge 2011).

Tortula ruralis (Hedw.) Gärtn., Meyer and Scherb. is a well-studied desiccation-tolerant moss (Green et al. 2011; Lakatos 2011). On sun-exposed rocks of a tropical inselberg in Brazil the desiccation-tolerant mosses *Campylopus savannarum* (C. Muell.) Mitt, *Racocarpus fontinaloides* (C. Muell.) Par., and *Ptychomitrium vaginatum* Besch. are subject to irregular water supply with drying and wetting cycles (Lüttge et al. 2008). Lichens dominate 8% of the terrestrial surface globally (Green et al. 2011). They are adapted to irregular water availability but not to too long periods of dryness. Regular diurnal cycles are typical with wetting by dewfall in the morning, fog in the afternoon, and dryness at noon. Nevertheless, lichens grow on bare rocks and even on anthropogenic substrates such as washed concrete in the temperate climate where the species *Lecanora muralis* (Schreb.) Rabenh., for example, is subject to drought, frost, and regular trampling (Lüttge 2013). Various reports mention survival times of desiccation in lichens of 2–4 months up to 51–56 weeks, and in one case even 5 years (Lange 1953, 1969; Green et al. 2011). On the washed concrete under dry weather conditions and with frost around −15°C during the night and high day-temperatures around 35°C, *L. muralis* recovered its photosynthetic activity from desiccation within 15 min after rewetting. A sample kept in dry air for 37 days recovered within 30 min and samples dried for 155 days took 300 min (Lüttge 2013).

The impact of extant bryophytes and lichens on their surrounding environment and the arising ecosystem services underline the likely role during early terrestrialization. The genetic basis was present. Among the extant bryophytes the liverwort *Marchantia polymorpha* L. and the moss *Phycomitrella patens* (Hedw.) Bruch and Schimp. have genomes providing the outfit required by ancestral land plants tolerating terrestrial stresses (Rensing et al. 2008; Bowman et al. 2017). Bryophytes are also important parts of biological soil crusts (Sect. 4.4), which through their thermal, hydrological, and carbon regimes link pedosphere and atmosphere within the biosphere (Green et al. 2011; Lakatos 2011). Due to carbon leakage, the crusts create microhabitats for fungi and support mycorrhizae (Lakatos 2011), which was already important for the early establishment of vascular plants on the dry land (Sect. 6.2.2).

4.4 Biological Soil Crusts

The likely early conquest of land by desiccation-tolerant cryptogams was assessed above. Cyanobacteria and green algae were coming from the water (Sects. 4.1.1 and 4.1.2) and bryophytes and lichens were early land plants (Sect. 4.3). Singly, cyanobacteria and algae can form thin biofilms and soil crusts of a few millimeters in width. This could have opened capacities for initiating the first wave of terrestrialization, succeeded by a second one between 420 and 410 × 10^6 years ago with the early vascular plants (Sect. 5).

However, early soil crusts may have already been more complex (Belnap et al. 2001a). Extant soil crusts can reach several centimeters of thickness. They are desiccation tolerant and cover any gaps with harsh conditions left open by Higher Plant vegetation. Biological soil-crust development extends across the world. Wide areas with a major development of soil crusts are the semiarid and arid lands which make up 30% of the Earth's surface. Thus, soil crusts cover a substantial portion of the globe (Belnap and Lange 2001). Following disturbance, crust organisms are the first to recolonize soil surfaces (Belnap and Eldridge 2001).

Soil crusts show a very high species diversity with bacteria, cyanobacteria and algae, lichens with cyanobacteria (cyanolichens) and green algae (phycolichens) as symbionts, bryophytes and fungi (Belnap 2001a; Büdel 2001). The presence of fungi (States et al. 2001) suggests that there may have been precursors of mycorrhizae in early terrestrialization by soil crusts, which later proved to be the essential symbiosis of vascular plants with fungi for the establishment of vascular plants on land (Sects. 5 and 6.2.2). In extant soil crusts mycorrhizal fungi are involved in increasing nutrient availability to plants (Belnap et al. 2001a, b).

Soil crusts contribute much to the carbon and nitrogen cycles and the hydrological balance of landscapes (Belnap 2001b; Evans and Lange 2001). They actively perform photosynthesis. Area-based maximum rates of net photosynthesis range from 0.1 to 11.5 μmol CO_2 m^{-2} s^{-1}. The higher rates correspond to those of needles of evergreen coniferous trees (4–8 μmol CO_2 m^{-2} s^{-1}), and some even come close to leaves of C_3 crop plants (20–40 μmol CO_2 m^{-2} s^{-1}) (Lange 2001). Given their worldwide distribution, crusts resemble a global "giant leaf" (Belnap and Lange 2001). Cyanobacteria are able to fix atmospheric N_2 (Sect. 4.1.1). Free-living and as symbionts in cyanolichens, they provide nitrogen to the ecosystems. Of the nitrogen fixed, 70% is immediately released to the surrounding soil environment (Belnap et al. 2001a, b).

Biological soil crusts are veritable ecosystems. They modify the soil environment in various ways (Belnap et al. 2001a, b), i.e., surface roughness and soil texture, thermal conditions, pH, availability of nutrients with essential elements such as N, K, Ca, Mg, P, Fe, Ni, Cu, Zn, Mn, Cl, S, organic matter, and water. In this way, niches are created for other life including a rich microfauna (Belnap 2001a). They influence vascular plants supporting germination, emergence, and survival (Belnap et al. 2001a, b). Therefore, we can speculate that after the first wave of terrestrialization soil crusts have facilitated the second one by vascular plants.

5 Conquest of Dry Land by Early Vascular Plants

In the following, we shall look at the early vascular plants and their establishment on dry land following Frey and Lösch (2004), Bresinsky et al. (2008) and Lüttge et al. (2010).

The oldest fossils we know of land plants in the late proterophytic geological time are those of *Cooksonia*, a genus which unfolded from the Upper Silurian to the Lower Devonian 420–410 × 10^6 years ago. It had about 10 cm high dichotomously branched rush-like shoots with a central protostele. It belonged to the *Rhynia*-group of the class Psilophytopsida of the fern allies (Sub-Division Pteridophytina). Besides *Cooksonia pertoni* Gonez and Gerienne other members of the group were *Rhynia gwynne-vaughanii* Kidst. and Lang, *Aglaophyton major* D. S. Edwards (syn. *Rhynia major* Kidst. and Lang (Fig. 1), *Stockmansella remyi* Schultka and Hass and *Horneophyton* spec., which altogether were sporophytes, and *Langiophyton mackiei* gametophyte of *Horneophyton lignieri* ((Kidst. and W. H. Lang) Bargh. and Darrah) and *Sciadophyton* spec., which were gametophytes. The name *Rhynia* comes from the Rhynie Chert in Aberdeenshire, Scotland, where the first fossils were found in the Lower Devonian. *Rhynia* had no leaves, no flowers, and no real roots. It had up to 1 m high dichotomously branched shoots with a central stele with tracheïds for long-distance transport of water which is essential for upright plants on dry land. Thus, *Rhynia* was a real vascular plant. It was anchored in the ground by horizontal rhizomes, and there were rhizoids which could have functioned in the uptake of water and dissolved minerals. It is most noteworthy that vesicular-arbuscular mycorrhizae are seen in the fossils of *Rhynia*. We assume that the establishment on dry land right from the beginning was not possible without this symbiosis with fungi facilitating the acquisition of water and minerals (Sect. 6.2.2). For the essential long distance transport of water *Aglaophyton* and *Horneophyton* had hydroids in their steles, as we also find them in the stemlets of mosses. The latter two genera still lacked tracheïds so that they were not yet vascular plants in a strict sense.

In the *Zosterophyllum*-group there were the gametophytes *Taeniocrada* and *Sciadophyton,* later recognized to belong to the life cycle of *Zosterophyllum* after having been first regarded as own genera. The genera *Gosslingia* and *Baragwanathia* also belong to this group.

The *Trimerophyton*-group comprised the genera *Dawsonites*, *Trimerophyton,* and *Psilophyton* which were already more developed. *Psilophyton princeps* Croft and Lang var. *ornatum* (syn. *Sawdonia ornata* J. W. Dawson) had small spikes along their stems possibly constituting early precursors of leaves.

The early vascular plants highlighted above are all extinct. They lived at the shore of lakes or in wet depressions. Some of them were amphibious. *Zosterophyllum* still grew in the water. *Cooksonia*, *Rhynia,* and *Psilotum* already were land plants. These psilophytes originally could have passed to the land by wind-borne spores released from shoots stretching out of the water or evaded from shore-near sites falling dry occasionally. The addressed vascular plants represent incipient terrestrialization.

Geological times among other points of view can be characterized by dominant plant life forms (Lüttge 2016), i.e.,

- proterophytic, Ordovician – Silurian, 510–409 × 10^6 years ago,
- palaeophytic, pteridophytes, Devonian – Carboniferous, 409–290 × 10^6 years ago,
- mesophytic, gymnosperms, Permian – Jurassic, 290–146 × 10^6 years ago,
- neophytic, angiosperms, Cretaceous – Tertiary, 146 × 10^6 years ago up to present.

Terrestrialization by vascular plants began in the late proterophytic and proceeded into the palaeophytic with the early pteridophytes. With this decisive step, we close our considerations within this review. Terrestrialization by vascular plants proceeded with remarkable speed in the Middle and Upper Devonian within just 40 × 10^6 years (Mägdefrau 1952; Lüttge 2017). Subsequently, as we know, EvoDevo of vascular plants created a plethora of adaptations to the terrestrial environment. Shaping that environment was an enormous success of plants during the subsequent ages of gymnosperms and angiosperms.

6 Stationary Life of Plants in Two Contrasting Environments: Pedosphere and Atmosphere

6.1 Two Central Questions

Two central questions are: (1) Why had the plants to go on dry land first? (2) Why are plants stationary? The answers to both questions are closely related. Only the primary autotrophic plants can pave the way and link the carbon and nutrient cycles of life. The biosphere is built up of life in water and on land and in the latter case of life in the pedosphere and in the atmosphere. On the land, the vascular Higher Plants live simultaneously in two environments with highly contrasting conditions, i.e., in the darkness of the mineral pedosphere and in the light of the gaseous atmosphere. This is possible by the bipolar gestalt of plants with roots and shoots. It was already anticipated in the evolution of filamentous algae, which were bipolar with rhizoid cells and growing tips (Sect. 3.3). For exploiting the pedosphere on dry land plants need to be stationary, i.e., anchored with their roots in the ground. The first vascular land plants of the psilophytes like *Rhynia* did not have roots yet (Sect. 5). Their settling on land resulted in an enormous environmental pressure for the evolution of roots to further advance terrestrialization.

6.2 *Pedosphere*

6.2.1 Plants and the Evolution of Soil

Plants play a primary role in mineral nutrition of the biosphere on the dry land of the Earth. To fulfill this role, they need to be stationary because at particular sites time is required for various very slow processes of weathering rocks for mobilizing mineral nutrients. One cannot imagine how this could be achieved by mobile life forms freely moving around. Soil did not exist on the dry land of Earth before being inhabited by life. Soils are complex vivid living ecosystems with microorganisms, fungi, the roots of plants, and small invertebrate animals. Besides the minerals, organic carbon compounds from excretions and dead matter of plants and animals are essential ingredients of soils. Envisaging the evolutionary advancement of soil we may consider extant processes.

Soil is formed by the weathering of solid bedrocks of the Earth's crust. We distinguish physical, chemical, and biological weathering. Physical weathering by wind, water, and extreme variations of temperature causes fragmentation of rocks. Loose parts of rocks are a starting point for soil formation. The chemical and biological weathering mobilizes minerals. Chemical weathering operates by inorganic processes. However, it is much enhanced by living organisms. The early terrestrial life on Earth with thallophytes interacting with their mineral substrates has contributed to soil formation. Production of carbonic acid from the CO_2 of respiration, as well as secretion of organic acids by the organisms, plays a major role in lowering the pH for dissolving mineral ions. Evolution of soil and terrestrial plants was interactive coevolution. Besides the CO_2 and organic acids plant roots secrete other organic compounds, such as sugars, amino acids, phytohormones, and phenolic substances, which determine soil ecology (Frey and Lösch 2004; Lüttge et al. 2010). The phenolic substances form so-called phytosiderophores, which specifically bind divalent and trivalent metal cations, e.g., calcium, iron, and manganese, in a chemical form suitable for uptake by the plants. Ions are also mobilized by ion-exchange adsorption based on overlapping electrical charge densities of the interacting surfaces of plant roots and soil constituents. Thus, via weathering of rocks, formation of soils, solubilization of minerals, and their uptake plants are the origin of funneling the essential minerals into the food webs of the biosphere. This is considered in this section regarding many metal cations, but it is specifically indispensable in the cases of nitrogen and sulfur as addressed below in Sect. 6.2.3.

6.2.2 Mycorrhiza

In a wide extended space of the soil plants only find access to the minerals by a mutualistic association with fungi. This symbiosis of plants and fungi is the mycorrhiza (Frey and Lösch 2004; Bresinsky et al. 2008; Lüttge et al. 2010). The heterotrophic fungi with their hyphae reach far in penetrating the soil and extending the exploitable space. So, they deliver water and dissolved nutrients to the plants. The autotrophic plants provide products of photosynthetic CO_2 assimilation to the fungi. Of all extant vascular plants 80–90% have mycorrhizae, which are absent only in the Brassicaceae and Chenopodiaceae. Mycorrhiza is even reported to occur in some mosses.

Mycorrhiza has participated already in the very beginning of the evolution of land plants. Without the mutualistic coevolution of fungi and plants, terrestrialization by vascular plants is unlikely to have been successful. Mycorrhiza must have played a decisive role for vascular plants to settle on land. The interaction with a vast network of symbiotic fungal hyphae in mandatory mycorrhiza must be stabilized. This required plants to be stationary fixed in the ground. Fungi present in the substratum of bryophytes (Sect. 4.3) and soil crusts (Sect. 4.4) can have been the partners in the early evolution of vascular plants. Fossils of the pteridophytic vascular plants found in the Scottish lower Devonian, such as *Rhynia* (class Psilophytopsida; Sect. 5) and *Asteroxylon* (class Lycopodiopsida), show that they had mycorrhiza. Such an original form of mycorrhiza, which is still very common, was the so-called vesicular-arbuscular mycorrhiza (VA-mycorrhiza). The hyphae of the fungi spread inside the roots of the host plants within the cell walls (endotrophic mycorrhiza). They generate vesicles, and tree-like branching haustoria are formed, resembling arbuscular structures giving the name to VA-mycorrhiza. With their large surface, the latter facilitates exchange of solutes between the partners.

6.2.3 Autotrophy of Plants for Carbon, Nitrogen, and Sulfur

The elements carbon, nitrogen, and sulfur are available in the inorganic environment in the oxidation forms of HCO_3^-/CO_2, NO_3^-, and SO_4^{2-}. They are needed in the organic compounds of biomass, however, in reduced form. Their reduction is a matter of autotrophy. Complete autotrophy means that the entire metabolism of an organism can be fed exclusively by inorganic materials. Only green photosynthesizing organisms are capable of this and stand at the basis of food webs.

The carbon cycle with primary autotrophic production of organic matter by plants is broadly appreciated. Photosynthesis is the well-known light-dependent fixation, reduction, and assimilation of CO_2. The mineral cycles have often been neglected, although especially the N-cycle is similarly important (Körner 2020). Only autotrophic photosynthetic organisms can reduce NO_3^- to NH_4^+, the oxidation state which is required for incorporation into organic matter. The essential enzymes are nitrate-reductase located in the cytosol and nitrite-reductase in the chloroplasts, where photosynthetic electron transport produces the reduction equivalents needed. Heterotrophic organisms obtain nitrogen from the biomass of autotrophic primary producers, and upon die-off return it in the food webs to remineralization by microorganisms. Putrefaction then first produces NH_4^+. Further interaction with microorganisms completes the N-cycles. Nitrification by bacteria converts NH_4^+ to NO_3^-, which plants reduce again to NH_4^+. In addition, some microorganisms by denitrification can convert NO_3^- to N_2. Some bacteria and most cyanobacteria can reduce atmospheric N_2 to NH_4^+. Similarly, in the S-cycles only autotrophic plants reduce SO_4^{2-} to S^{2-} in their chloroplasts. Some bacteria can de-sulfuricate SO_4^{2-} to S^{2-}, while others can sulfuricate S^{2-} to SO_4^{2-} (see Lüttge et al. 2010).

6.3 Atmosphere

Plants are not only the basic primary producers of biomass for the food webs transforming inorganic precursors into organic matter. Living in two contrasting environments they also shape the pedosphere with soil and, in parallel, the atmosphere with its major gases CO_2, O_2, and water vapor. Via photosynthesis, plants consume CO_2 and produce O_2, and via transpiration they release water vapor into the atmosphere.

6.3.1 Carbon Dioxide, CO_2

In the early atmosphere of our planet carbon dioxide was the main gas besides water vapor. Its partial pressure was orders of magnitude higher than the current about 4×10^{-4} bar at a total atmospheric pressure of 1 bar. In the proterophytic, 500×10^6 years ago, it was still 20 times, and at the end of the proterophytic 400×10^6 years ago, when the first vascular plants established themselves on land, it was 15 times the current level, i.e., about 0.6%. Carbon dioxide is the essential substrate for the primary production of biomass by plants. As CO_2 is a greenhouse gas, this is relevant in the discussion of present global warming, but in terrestrialization CO_2 was a target rather than a driver. This is completely different for O_2.

6.3.2 Oxygen, O_2 and O_3

The early atmosphere was free of O_2. Oxygen was produced by the first organisms performing oxygenic photosynthesis about 3×10^9 years ago. It built up in the atmosphere only slowly because it was bound in the oxidation of reduced minerals in the Earth's crust, particularly iron. The O_2 level in the atmosphere $2–1.5 \times 10^9$ years ago was 0.2%. Initially, O_2 was an aggressive oxidizing poison, but when – presumably as an adaptation – respiration evolved, O_2 became essential for life. It was an absolute prerequisite for terrestrialization by heterotrophic animals following the terrestrialization by plants (Layer and Lüttge 2020).

During the evolution of O_2 levels in the atmosphere, there were large variations and there prevail many gaps in our knowledge about that (Layer and Lüttge 2020). However, major trends and their importance for terrestrialization are clear. About 630×10^6 years ago, there was a steep increase in O_2 concentration. During much of the proterophytic it was about 12%. At the end of it, in the late Silurian when terrestrialization by vascular plants began, it had reached 15%, while the CO_2 concentration was still about 0.5%. The very dense photosynthesizing vegetation in the Carboniferous became effective about 285×10^6 years ago, when the atmospheric O_2 concentration increased to 35%, while the CO_2 concentration declined to present levels at about 0.04%.

With respect to terrestrialization oxygen was not only decisive for the respiration of the heterotrophic life of animals. It played a dual role. Together with the increase of atmospheric O_2 during the proterophytic a stratospheric ozone-shield, O_3, was built up. This protects from solar ultraviolet radiation including the very dangerous DNA-injuring UV-C. That shield was essential for the establishment of all life on land, which otherwise would not have been possible outside the water.

6.3.3 Water Vapor, H_2O

All organisms to a large percentage of their fresh mass, often more than 90%, consist of water. Water must be available for life on land. The various resources are fed by precipitation. The level of H_2O vapor in the Earth's atmosphere is variable between 0 and 4%. Plants contribute to air humidity by transpiration, the loss of water vapor concomitant with the uptake of CO_2 in photosynthesis. As a consequence, the plant-cover contributes to cloud formation and precipitation. Water vapor is also the major greenhouse gas. Without the water vapor in the atmosphere the mean air temperature on Earth would drop to -18 C, being too low for life.

7 Directed Evolution from Aquatic to Terrestrial Life: Teleology

Thinking of directedness is banned in exclusively chance-based Neo-Darwinism. Directedness implies teleology. An explanation is considered teleological if purposes and aims are understood as constituting the explanatory factors. Such purposes could include intrinsic natural properties of things (Mahner 2018). From the point of view of the metaphysics of ontological materialism or naturalism, teleology is forcefully rejected (Mahner 2018). When teleological explanations are identified with scopes of intention, as in the philosophy of Mahner (2018), and are used in creationism or even in attempts to prove the existence of God, we fully agree with him to reject them. We must maintain the methodological atheism or agnosticism (Ebertz 2017), or a non-interventionalism (Scherer 2017). However, restricting teleology to intention appears as a too narrow view of it.

Directed EvoDevo is more than ontogenesis from zygote to organism. Ontogenesis is genetically steered by environmental constraints. The phenotype is not a firmly fixed mirror picture of the genotype so that we need to modify Neo-Darwinism. Preadaptations, such as desiccation tolerance and lignin, and especially the stretched bipolar growth form among algae for a life on land, have a teleological character in a sense that directed evolution is supported. Terrestrialization might be an outstanding example of directed evolution. This has nothing to do with intention. It does not exclude the participation of chance. However, chance is not necessarily effective via initial mutation followed by environmental stress and selection. The process may ensue environmental changes with permanent, but not yet genomically fixed inheritable adaptation, which in turn may support mutations. Depending on environmental conditions, development can be variable but not ad libitum. Choice is controlled by the directive restrictions of devices available in toolboxes. We distinguish a molecular and a morphological toolbox (Layer and Lüttge 2020). The former comprises very ancient molecular mechanisms and genes on stock which have prevailed throughout evolution, and which by gene duplications and modification of function contributed to diversity. The morphological toolbox contains structures. In early evolution, for example, during the Cambrian explosion, life forms were tested vigorously by the prevalent environments during the process of selection. Eventually selected structures persisted in the morphological toolbox. In such a way, the toolbox continually embraced preadaptations of potential evolutionary value under subsequent environmental scenarios.

The outlined tendencies are also reflected by the phenomenon of convergence. In response to specific environmental pressures phylogenetically widely separated lineages developed very similar structures, as emphatically advocated by Simon Conway Morris (2003, 2008). Noteworthily, the title of the German translation of his book is “Jenseits des Zufalls” (“Beyond chance”, Morris 2008). Convergence is ubiquitous and dominates life. Examples in the book of Morris mainly from animals but also from plants are numerous. They include the molecular level, where DNA and the genetic code, and chlorophyll are noteworthy. The number of evolutionary solutions to specific biological and ecologically mediated challenges evidently is often quite limited. Conspicuously, the number of theoretical basic “blueprints” towards building plants and animals and forming gestalt is restricted. In such terms, the many examples of convergence eminently mirror some kind of directedness.

Selection of preadaptations from the toolboxes has a teleological touch. We must also note that epigenetic modifications of gene expression by states of methylation and acetylation without affecting the sequences of bases in the DNA of the genetic material can store responses to external conditions, such as nutrition, climate, population density, stress, epidemics, etc. (Boyko and Kovalchuk 2008; Chinnusami and Zhu 2009; Adams 2010; Kinoshita and Seki 2014). These epigenetic modifications can be inherited to subsequent generations (Jablonka and Lamb 1989; Bird 2002; Kakutani 2002; Molinier et al. 2006; Bond and Finnegan 2007; Saze 2008) and direct EvoDevo.

The drive of conquest of space operating with selection of available devices from the toolboxes of preadaptations can be determined both by chance under variable environmental stress and by EvoDevo associated directedness. The latter must not be overlooked, as the process of terrestrialization by flora and fauna may appear as a most relevant example (Layer and Lüttge 2020).

8 Conclusion

Three eminent questions are asked in this essay. Why was there terrestrialization at all? Why had green plants to go first before heterotrophic animals? Why need plants to be stationary? Answers to the last two questions are embedded in the biospheric services of plants linking pedosphere and atmosphere. An answer to the first question is seen in the drive for occupation of any possible space, which appears as a fundamental and, therefore, intrinsic property of life. This drive explains that terrestrialization was a necessity. Such a drive is also seen in the behavior of mankind itself, whether it was the exploration of the Antarctic and the Moon and now is the dream of occupying Mars. The latter step may not be a principally more dramatic challenge than it was for a unicellular alga to get out of the water and establish itself on bare rocks of the dry land. The use of the available preformed toolbox and its further evolution adjust to primarily adverse conditions. The self-organization of life, exemplified by the step of terrestrialization, is conducive of thinking of directed evolution under an EvoDevo perspective (Layer and Lüttge 2020). The drive for occupying space we clearly recognize as an intrinsic property of life, although we do not understand it. This is similar to other limits of knowledge about life. We do not know how life originated. We cannot delineate and define life and only describe it.

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Legacies of Human Land Use Impacts in Central European Forests

M. Wulf

Contents

Abstract Forests are generally considered relatively near-natural ecosystems, despite often experiencing fairly intensive uses, including conversion of the main tree species and intensive management. In Central Europe, it can be assumed that forest with continuity of thousands of years remained only in locations inaccessible to humans (e.g., steep slopes in remote mountains). For most other forested areas, it is obvious that they were completely or partly deforested at least once in the past and used as non-forested land (e.g., arable land or grassland). The wide distribution of historical arable fields (e.g., Celtic fields or ridges-and-furrows) throughout Europe is the best confirmation of this belief. Particularly in the last two decades, the long-lasting effect of previous non-forest uses on patterns and processes of abiotic and

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biotic parameters of currently forested areas has been studied more intensively. In parallel, the research field of historical ecology, which is not new in itself, has received increased attention and is considered significant to the further study of ecosystems and landscapes. Due to the large number of existing publications, it is possible to summarise the results to date and to identify research gaps that still exist. This information was compiled and is contained in the five sections in this paper. Definitions and procedures are presented in Sect. 2, which is divided into subsections on the definition of landscape legacies, human interventions and time frame establishment (Sect. 2.1) and the search and selection of publications (Sect. 2.2). The findings on aboveground and belowground legacies (Sect. 3) are presented separately for vascular plants and cryptogams (Sect. 3.1), animals or animal communities (Sect. 3.2), topographic features (Sect. 3.3), soil chemistry and soil biological activity (Sect. 3.4) and biomass and tree growth (Sect. 3.5). In Sect. 4, these issues are discussed in connection with research questions that have not been answered or have only been answered incompletely. Finally, essential conclusions are concisely provided (Sect. 5).

1 Introduction

At least 90% of the landscape of Central Europe would have covered naturally by forests, if they had been continuously maintained (Bohn and Neuhäusl 2000/2003). Among the terrestrial ecosystems of this region, forests are therefore considered natural or near-natural. However, there is plenty of evidence that there were large periods of deforestation starting in the twelfth and thirteenth centuries until the seventeenth century, so that ultimately a significant reduction in the original forest cover occurred (Pongratz et al. 2008; Fuchs et al. 2015). Forests are still an important landscape element today, currently covering 37% of the land area of Central Europe (according to data in Parviainen et al. 2000).

Today's forests, however, are not remnants of the original forested areas, as numerous traces of previous non-forest uses indicate. The first and foremost are the so-called Wölbäcker or ridges-and-furrows, which were widespread throughout Europe in the Middle Ages (Alcántara et al. 2017) and are still clearly visible in the forests of some regions (Trächsel 1962; Noack and Wulf 2019). Second, there is evidence of former agricultural uses dating back even further, the so-called Celtic fields, which are limited to northwestern Central Europe (Arnoldussen 2018). Third, even in more remote and sparsely populated landscapes than Central Europe, evidence of past land uses, such as traces of Neolithic farmland in the boreal zone of Northern Europe (Alenius et al. 2013) or "field systems" in the Amazonian forests (McKey et al. 2010; Maezumi et al. 2018), has been found. In fact, it can be assumed that the vast majority of today's forested area in Central Europe was used as farmland at some point in the past.

Thus, the present forested area has been created either by plantings or succession after previous non-forest uses, and these processes may have occurred repeatedly since the retreat of glaciers roughly 14,000 years ago. Furthermore, even forested areas that have existed for a long period have often been used relatively intensively for a very long time, e.g., through forest pasture or litter raking (Gimmi et al. 2013; Leuschner and Ellenberg 2017). The dominance of oak over beech in many Central European hardwood stands is due to the centuries-long tradition of wooded pastures, and species-poor lichen-pine forests are the result of at least decades of litter removal and thus severe nutrient depletion of the topsoil. These are just two examples of the numerous legacies of former forest uses in Central European forests that are still recognisable or detectable decades or even centuries later (Leuschner and Ellenberg 2017 and references therein).

The long-lasting effects of former human uses have resulted in the terms “ecological memory” of landscapes or ecosystems and “landscape memory”. Important contributions to this topic have been provided by Brierley (2010) and Ogle et al. (2015) in principle, and Wieland et al. (2019) have addressed this topic with a big data approach. The discipline of “historical ecology” is very closely linked to the concept of ecological memory. This field of research is not new but has long been anchored in four scientific disciplines, namely, anthropology, ecology, geography and history (Bürgi and Gimmi 2007; Szabó 2015). Although the approaches of these disciplines can vary greatly, all authors agree that studies on historical ecology, especially when they concern patterns, events and processes of the past, contribute to a better understanding of the present (Szabó 2015). In fact, numerous landscape ecology studies have shown that present patterns or processes in forests are often better interpretable, when historical events or influences are taken into account (e.g., Grashof-Bokdam and Geertsema 1998; Hermy and Verheyen 2007; Gimmi et al. 2013; Bürgi et al. 2017).

The discipline of historical ecology has experienced new interest due to improving remote sensing (LiDAR) technologies, resulting in digital terrain models (DEMs), especially in the last two decades (see Fig. 2 in Szabó 2015). High-resolution methods have allowed “old traces” in landscapes to be identified and becoming visible for large areas (Roman et al. 2019). For example, technological progress has made it possible to detect the extent of the former distribution of Celtic fields in northwestern Central Europe (Arnoldussen 2018), whose remnants are most likely still found in forests, and the multitude of charcoal kilns in forests in southern Brandenburg (eastern Germany, Raab et al. 2019) could be demonstrated. In addition, the increasing digitisation of historical maps has significantly contributed to the depiction of changes in land cover over longer periods of time for numerous landscapes (Verheyen et al. 1999; Glaser and Hauke 2004). This information provides a basis for distinguishing forested areas with previous non-forest uses (often termed post-agricultural forests) from those with long habitat continuity.

The importance of forests with long habitat continuity, especially those that exist for at least 200 years (so-called ancient forests; details in Sect. 2.1), for certain animal and plant species has been extensively documented (Peterken 1993; Nordén et al. 2014). There are now also several studies that show how interim non-forest uses still affect the occurrence of species or the characteristics of communities in forests decades later (Hermy and Verheyen 2007; Bergès and Dupouey 2021). These observations, which have also been made in other non-forest ecosystems, have led to the concept of legacies. Fraterrigo (2013) uses the term landscape legacy, which has the same meaning as the ecological memory or landscape memory introduced above (for terminology, see Sect. 2.1).

The recently published review by Bergès and Dupouey (2021) summarises many general findings on land-use legacies in forests. However, the authors limit their review to vascular plants, while excluding studies on animals and topographical features, and only refer to a few publications from the field of soil science. This present review adopts a broader perspective by including a range of abiotic factors and addressed both vascular and non-vascular plants, macro-fungi and animals to examine the role of legacies for the composition of biotic communities in Central European forests and important ecosystem processes. The main objectives are (1) to provide a comprehensive overview of published studies and to compile them in publicly accessible files, (2) to summarise the most important results, (3) to identify research gaps and (4) to discuss important questions for future research on the topic.

2 Definitions and Methodology

2.1 Definition of Legacies and Considered Time Frames

In the definition of legacies, this review follows Fraterrigo (2013), who defines landscape legacies as “the long-term impact of historical events on ecosystem structure, composition, or function” (Fraterrigo 2013, p. 524). He includes natural disturbances (discrete events not directly caused by humans) and land use by humans as historical events. The terms “long-term” and “historical” are not defined in detail by Fraterrigo (2013), but he repeatedly mentions events in the distant past and their effects that are still detectable decades to centuries later.

In this review article, I do not address natural disturbances such wind throw or fires. One reason is that legacies related to natural disturbances are much less studied than those related to direct human interventions (Bergès and Dupouey 2021). In addition, Fraterrigo (2013) raises the concern that natural disturbances can have potentially significant, long-lasting effects on ecological patterns and processes in temperate forests, but the resulting dynamics are in Central Europe frequently constrained by patterns caused by past land uses. Therefore, this review only considers studies of previous human land uses that resulted in a prolonged interruption of canopy cover. These land uses include former settlements, gardens, arable fields, grasslands or heathlands. In the following sections, the legacies of these non-forest uses are referred to as human land-use legacies or just legacies.

Since Fraterrigo (2013) does not define historical events or the distant past in more detail, the question of which time periods are meaningful remains. In this context, it is also important to define forests with long habitat continuity. In 1993, an international conference was held by the North German Academy of Nature Conservation on the “Importance of the age of biotic communities”, using the example of “historically old forests” in Lower Saxony (North Germany) as a study object.

During the conference, the following definition was agreed upon: “Ancient forests are forests on forest sites which, according to evidence from historical maps, stand descriptions or other indications, have existed continuously for at least several hundred years” (Wulf 1994). In Central Europe, forests with at least 200 years of habitat continuity are usually referred to as ancient forests, and this definition takes into account the fact that historical maps for large parts of Central Europe are available from approximately the middle or end of the eighteenth century. In the UK, however, ancient forests are referred to as forests with a habitat continuity of at least 400 years because the available archival records go back further.

In principle, ancient forests are referred to in this article as forests with a habitat continuity of at least 200 years and those included studies that can be clearly classified as ancient forests either by historical maps or by stand age (if author (s) mentioned that several trees are at least 200 years old). However, a few papers have been included where the habitat continuity of forest patches is somewhat shorter to avoid withholding essential findings on legacies in present-day forests. These studies are marked in Table A in the Appendix, which contains all studies that were included in the review.

It is also important to clarify how long ago historical events should have occurred to be classified as historical. On this point, I follow the statement in Bergès and Dupouey (2021) on page 11, who mentioned human land uses dating back at least 50 to 100 years. That means, studies were considered where the previous non-forest uses date back at least 50 years. However, few exceptions were made to ensure sufficient data base (marked studies in Table A).

In order to imprint on forest structure, the duration of the former land uses should have occurred over at least a few decades, but the vast majority of studies do not provide any information on this topic. Nevertheless, it can be assumed that fields or grassland areas that were once laboriously cultivated were not used as such for only a few years and then converted back into forests. This consideration is certainly also true for former settlements, including associated gardens or other economic areas close to settlements. Forests with such previous land uses are termed recent forests or post-agricultural forests.

The most far-reaching evidence of former non-forest uses is related to Celtic fields from the Iron Age/Bronze Age, and settlements dating back to Roman times, i.e., to approximately 50–250 AD. For the period thereafter, medieval ridges-and-furrows, which were probably established often in the twelfth century but regionally also as early as the seventh and eighth centuries, are worthy of mention, as well as charcoal kilns, the earliest dating of which is in the sixth and ninth centuries. For the more recent period from approximately the middle/end of the eighteenth century onwards, historical maps are available for large parts of Europe that can provide information about the former land uses, but there are also numerous other historical sources.

2.2 Search for References and Their Selection

The majority of the references used here were obtained through searching the ISI Web of Science database, using the word combinations “temperate forests and legacies” or “European forests and legacies”, and Google Scholar, using the terms “legacies in temperate forests” or “legacies in European forests”. The literature was supplemented by own databases, built over 30 years, and research within the reference lists in existing publications.

The studies finally evaluated were selected according to the following criteria:

1. Preferably, the studies were published, peer-reviewed and written in English, but at least, they were available on the Internet.
2. Non-forest use must be proven as a prior use of the current forested area.
3. The previous use should have occurred over at least several decades; if the duration was not specified, then the descriptions of the study area needed to convincingly show that the criterion was met.
4. To not reduce the data or knowledge base too much for some aspects due to a too small number of available studies, a few studies were considered whose previous uses dated back less than 50 years. Notes are included in the text for these studies, and the studies are indicated in Table A.

A total of 202 selected studies are listed in Table A and mentioned in the text. Among these, some 21 have investigated more than one of the aspects listed in Sects. 3.1–3.5, resulting in 181 studies in total:

On vascular plants, cryptogams and plant communities	103 studies
On animals or animal communities	26 studies
On topographical features	28 studies
On soils	36 studies
On tree biomass, growth and soil microbial activity	9 studies

3 Above- and Below-Ground Legacies of Human Land Uses

3.1 Vascular Plants and Cryptogams

Overall, the largest number of studies on the legacy of former non-forest uses in present-day forests was carried out on vascular plants or plant communities and cryptogams (Table A). For these groups of organisms, several lists of ancient and recent forest indicators have been compiled (Rose 1976a, b; Honnay et al. 1998; Wulf 1997; Rose 1999; Schmidt et al. 2014). A key research question was whether recent forests harbour lower plant species numbers than ancient forests. The number of studies where more species occurred in ancient forests than in recent forests, or vice versa, or where the total number of species was almost equal, balances out. In some cases, a higher number of species in ancient forests can simply be explained by forest area and thus the species-area relationship (Jacquemyn et al. 2001), but there are also cases where a positive species-area relationship does not show up due to a more intensive management in larger forest areas (for example, Lawesson et al. 1998); in such cases, the number of species is not related to habitat continuity.

Among the studies in which the species numbers in recent forests were higher than those in ancient forests, it is striking that most of the previous uses were settlements, some of which dated back to Roman times (Dupouey et al. 2002; Dambrine et al. 2007). The small evergreen *Vinca minor* L. is considered an indicator plant of former Roman settlements (Prange 1996), and the strong association of *Gagea spathacea* (Hayne) Salisb. with ancient forests is associated with prehistoric cultivation (Celtic fields; Arnold and Fichtner 2018). It is also notable that in cases where the total number of species did not (significantly) differ, either the previous use dated back quite a long time (at least seven decades) or the ancient and recent forest stands were located within a forest complex, i.e., recent forests were not isolated, which usually resulted in more favourable conditions for colonisation (Bossuyt et al. 1999b; Bossuyt and Hermy 2000; Wulf and Heinken 2008; Dittrich et al. 2013).

Very often, ancient forests differ from recent forests in terms of community composition or with respect to certain species groups. In almost all studies listed in Table A concerning Querco-Fagetea communities (i.e. the class of Euro-Siberian deciduous forests), ancient forest indicators and rare or endangered species were only found in ancient forests. Quite a few studies showed that locally or regionally rare or endangered species occurred exclusively or predominantly in ancient forests (Brunet 1993; Brunet and von Oheimb 1998; Wulf 2004a; Hofmeister et al. 2019). In a few studies, more herb species were found in recent forests than in ancient forests (Dzwonko and Loster 1988; Sciama et al. 2009), and facultative forest species with main occurrence in non-forest vegetation were usually predominant in recent forests (Hermy and Stieperaere 1981; Dzwonko and Loster 1988; Wulf 2003, 2004b).

In comparison with recent forests, several studies observed a higher overall cover of the herb layer or higher abundances of individual species in ancient forests (Dzwonko 1993; Petersen 1994; Graae 2000; Gossner et al. 2008; Matuszkiewicz et al. 2013), including one of the most widespread species in European forests, *Anemone nemorosa* L. (Brunet 2004). This species was considered an indicator species for ancient forests in many European countries (see Hermy et al. 1993 and references therein). Other examples included *Hedera helix* L., which occurred significantly less often on former heathlands, and *Maianthemum bifolium* L. and *Vaccinium myrtillus* L., which occurred less often in forests established on former meadows (Grashof-Bokdam and Geertsema 1998).

The most plausible explanation to date for the significantly more frequent or exclusive occurrence of certain plant species in ancient forests is the lack of effective modes of long-distance dispersal in these species (Hermy et al. 1993; Verheyen et al. 2003c; Nordén et al. 2014; Kowalska et al. 2017). Thus, species that are dispersed in ancient forests by ants (myrmecochores) are autochorous or produce only few heavy seeds, usually lack in recent forests (Graae and Sunde 2000; Kimberley et al. 2013). While the diaspores of most forest plants form a transient seed bank (Graae and Sunde 2000), Plue et al. (2008) found a higher total seed density in ancient forests than in recent forests (former Gallo-Roman sites), irrespective of whether *Juncus effusus* L. was included or not. However, the total species richness and the Shannon-Wiener diversity index of the diaspore bank were significantly higher at the Gallo-Roman sites (Plue et al. 2008). Similar results were found by Bossuyt and Hermy (2001) who analysed 36 seed bank studies from 20 publications. In comparison with former grassland sites, former heathland sites had a higher seed density of open-land species, and these grassland sites had a higher seed density of these species than of taxa from ancient forests. The effect of former land use decreased after 50 years due to declining seed viability; i.e., the total seed density decreased with the age of the recent forests.

Another characteristic of plant species in ancient forests is their mostly vegetative propagation (Graae and Sunde 2000), and these plant species are often early flowering plants (geophytes, Kelemen et al. 2014). In ancient forests of the Querco-Fagetea class, competitors according to Grime's (1988) plant strategy classification are the dominant type (Brunet 2004; Wulf 2004a; Bergès et al. 2017; Abadie et al. 2021), while in recent forests, stress-tolerant species generally predominate (Vanwelleghem et al. 2004; Wulf 2004a). In addition, Abadie et al. (2021) found more frequent therophytes on former arable land and more frequent chamaephytes on former pastureland.

However, two further aspects of different species numbers or species compositions in ancient and recent forests should not be ignored. One aspect is a widespread use, known to occur since the Stone Age, namely the use of coppice or coppice-with-standards (Leuschner and Ellenberg 2017). Coppice forests were mainly established to address the demand for firewood (Szabó et al. 2015; Hédl 2018), and this was ensured by regularly cutting back sprouting woody plants at intervals of approximately 10–20 years. This repeatedly led to a massive disturbance to the stand structure, which initially resulted in a strong thinning of the stand, only to darken the herbaceous layer more strongly again due to the numerous sprouting woody plants. In the case of coppice-with-standards, these interventions were clearly reflected in the diameter growth of the standards, usually oaks (Altmann et al. 2013). The use of coppicing was still widespread in Europe in approximately 1600 AD, but declined in the nineteenth and twentieth centuries, and it is now rarely or no longer used even in countries where it was once widespread, such as Spain and parts of Denmark (McGrath et al. 2015). The consequence of abandoning coppicing, notably the decline in species supported by this practice, is increasingly being studied in recent times (Kopecký et al. 2013). It has become clear that traditional management of coppice and coppice-with-standards contributed significantly to the species richness of these forests by favouring disturbance-tolerant and light-demanding species.

Another aspect becomes clear from the study by Perring et al. (2018), which focuses on continuously forested areas (ancient forests) that differ in their former management practices (high vs coppice forests). Remarkably, against the background of higher N deposition, there was an increase in species richness and plant height in forests that were less intensively managed in the 1800s (high forests), but a decrease in richness in forests that were intensively managed in the 1800s (coppice forests) occurred.

The contrasts between ancient and recent forests are more obvious when comparing isolated ancient forests with isolated recent forests. This was demonstrated for plant species number by Peterken and Game (1984). The authors were able to show that in both types of forest, the number of species increases with the area but that the number of species in the ancient forests was higher than that in the isolated recent forests. Subsequent studies of recent forests directly bordering ancient forests repeatedly showed that the number of typical forest species and species dispersed by ants decreased with increasing distance from the ancient forest (Brunet and von Oheimb 1998; Bossuyt et al. 1999b; Verheyen et al. 2003b; Wulf and Heinken 2008; Orczewska 2011). Thus, the longer a recent forest has existed directly adjacent to an ancient forest, the more closely the plant communities converge. Depending on site conditions, the period needed for community recovery ranged from approximately 70 to 120 years (Verheyen et al. 2003a, b), because species reach distances of approximately 30–40 cm/year (slow coloniser) up to 3.0–11.5 m/year (fast coloniser) (e.g., Brunet and von Oheimb 1998; Brunet et al. 2000, 2012). Studies outside Europe have drawn the same conclusions (e.g., Matlack 1994). If no differences between ancient and recent forests have been observed (e.g., Kühn 2000), then a long period of re-colonisation may be an explanation for this observation.

In two studies, higher average specific leaf areas (SLAs) were measured in the communities of recent forests than in ancient forests, which was explained by an increased occurrence of shrubs or shrubs and trees in recent forests (Sciama et al. 2009; Bergès et al. 2017).

Bryophytes, lichens and macro-fungi (hereafter only fungi) have not been studied as frequently as vascular plants, but there are enough studies that mention their high affinity to forests with long habitat continuity (Rose 1976a, b; Tibell 1992; Mölder et al. 2015; Wirth et al. 2019). However, it is not always clear in these studies whether stand age or the presence of old trees and associated microhabitats is more decisive than the habitat continuity of the forest in determining this affinity (e.g., Tibell 1992; Gauslaa 1995; Rolstad et al. 2002; Jüriado et al. 2003). The problem becomes particularly clear, when only mature and old stands are compared and not stands that are comparable in terms of the main tree species and structure but with different continuity degrees, i.e., ancient versus recent forest. These and other points of criticism have been sufficiently raised by Lõhmus and Lõhmus (2011, note that they compare old growth with mature stands), which is why a reference to their study is included. In contrast, this review focuses mainly on studies that have directly compared ancient and recent forests. Other studies were not categorically excluded but were mentioned with appropriate related information. Some of these other studies present lists of bryophytes, fungi and lichens as indicators of ancient forests (Rose 1976a, b, 1993, 1999; Gustafsson and Hallingbäck 1988; Gauslaa 1995;

Fichtner and Lüderitz 2013; Whittet and Ellis 2013; Mölder et al. 2015), but the studies were not all based on a systematic comparison of ancient forests with recent forests.

A study from the Czech Republic is notable, as a generally positive relationship was observed between the richness of vascular plants as indicators of ancient forests and other forest-dwelling taxa, i.e., bryophytes, fungi and lichens (Hofmeister et al. 2019). Other studies showed a higher number of bryophytes in ancient forests than recent forests (Humphrey et al. 2004; Bergès et al. 2017), but this did not apply to lichens. In contrast, Fritz et al. (2008) found significantly more lichen species, but not bryophytes, in ancient forests. A higher number of lichens in ancient forests than in recent forests has further been confirmed in a relatively large number of studies from different regions (Tibell 1992; Jüriado et al. 2003; Vogt-Schilb et al. 2018), with one study pointing out that in comparison with "eastern-distributed indicator species", "western-distributed indicator species" tend to be more strongly associated with forests of long continuity (Whittet and Ellis 2013). Higher species numbers were also found for fungi in ancient forests than in recent forests (Hofmeister et al. 2014; Vogt-Schilb et al. 2018). Significantly fewer studies report no effects of habitat continuity on cryptogams (Dittrich et al. 2013) or no affinity of lichens and (calicioid) fungi with ancient forests (Ohlson et al. 1997; Lõhmus and Lõhmus 2011, but see note above).

3.2 *Animal Species and Animal Communities*

The number of studies on animals in relation to forest continuity is relatively few (Table A), which is surprising in that animal groups and individual species with a strong affinity to ancient forests have been reported from various regions of the UK for several decades (Peterken 1993, references therein) and also from other countries (e.g., Müller et al. 2005). One reason may be the labour effort needed to analyse invertebrate communities in forests. The list of publications focusing on animals becomes even shorter, when stricter criteria are applied, because several field observations in the identified studies were not based on a systematic comparison of ancient and recent forests. Nevertheless, not all studies have been disregarded here, as they provide valuable information that can be compared to the plant studies.

The vast majority of studies on insects, and the very small number of studies on other animal groups, such as birds and mammals (Table A), are striking. In addition, the significantly larger number of studies on aboveground rather than on belowground animals is remarkable. Despite these limitations, some clear trends can be identified.

Among the aboveground animals, beetles in particular are well studied. Their numbers were higher in ancient forests than in recent forests, and this scenario is especially true for those species that are not well adapted to disperse over long distances (Aßmann 1999). Such stenotopic forest species were found mainly or exclusively in ancient forests (Dekoninck et al. 2005; Desender et al. 2005; Buse

2012) and the abundances and population sizes were greater in ancient forests than in recent forests (Desender et al. 1999). Certain species had a higher genetic diversity in larger than in smaller ancient forests, e.g., *Carabus problematicus* (Desender et al. 2004; Huhta and Ojala 2006). These differences are generally more pronounced in isolated ancient and recent forests, such as occurs for vascular plants, than in larger non-fragmented forests consisting of a mosaic of ancient and recent forest stands. Here, ground beetles did not show any preference for one or the other forest type (Hülsmann et al. 2019).

Hoverflies occur with a higher total number of species (Proesmans et al. 2019) and a higher number of forest specialist species (Herrault et al. 2016) in ancient forests than in recent forests. Other studies found a higher diversity of hoverflies, but significantly lower proportions of forest species among true bugs, in recent forests than in ancient forests (Dekoninck et al. 2005; Gossner et al. 2008). However, Humphrey et al. (2004) questioned the extent to which the increase in Coleoptera species with an increase in per cent cover of semi-natural forests was actually based on an effect of habitat continuity. It is equally possible that this species group simply survived in recent semi-natural forests, old hedges, meadows or brackens.

For a region in Romania, Culbert et al. (2017) showed that bird populations varied significantly between ancient and recent forests without considering individual species. However, they found no effect of habitat continuity for butterflies. In Sweden, on the other hand, it was observed that the diversity of butterflies in a current old coniferous forest was higher than that in the area of former clear-cuttings, which were otherwise continuously used as forests. Remarkably, the former grassland use of the forest area dates back 70–90 years (Ibbe et al. 2011). The increased diversity of hoverflies in a recently afforested region in Belgium is less surprising, given that the forest has grown only approximately 25 years since the area was in a non-forest use, which is quite short. Dekoninck et al. (2005) observed a decrease in open-land species in favour of typical forest species, but even after almost 25 years, these species were only present in small numbers, including very few rare species typical of ancient forests.

The only known related study on mammals is from the UK (Bright et al. 1994). Despite comparable habitat qualities enabling the occurrence of the dormouse (*Glis glis*), it was found more frequently in ancient forests than in recent forests. The authors see this as confirmation of an earlier assumption; namely, the species has a patchy distribution.

Among soil animals, a higher diversity of earthworms in recent forests (up to 50/60 years) than in ancient forests has been documented by two studies from Belgium and England (Muys et al. 1992; Ashwood et al. 2019). In birch stands that were used as an arable field a few decades earlier, the populations of soil mites were generally smaller than those in birch stands that were previously stocked with spruce or birch (Huhta and Niemi 2003). However, no differences in the macroarthropod populations were found in the same areas (Huhta 2002), and Janssen et al. (2018) also found no effect of habitat continuity for springtails. The reason may be that the forests were previously used as arable fields, meadows and pastures 70–100 years ago. In Belgium, no differences in aboveground invertebrate community composition were found between ancient and recent forests; however, population sizes were significantly larger at post-agricultural sites, and plants suffered more from herbivory. Root-feeding nematodes also showed higher abundance at the post-agricultural forest sites than in ancient forests. Based on nutrient analyses in soil and plants, it is likely that the increased foliar P content (and only to a lesser extent N content) made the plants more susceptible to herbivore attack (De La Peña et al. 2016).

3.3 Topographical Features

3.3.1 Celtic Fields

Celtic fields date back to the late Bronze Age or pre-Roman Iron Age and are arable fields divided into generally rectangular or square fields (approximately 35 × 35 m up to 50 × 50 m in size) by wide and flat ramparts (Arnold 2011; Creemers et al. 2011). They have been well studied, especially with regard to their chronological classification and function (Bradley 1977; Müller-Wille 1979).

Recent studies have primarily focused on the discovery of Celtic fields because they can now be visualised via LiDAR allowing higher resolution for large areas (Kooistra and Maas 2008; Humme et al. 2013). Studies have shown that arable fields over 2,000/2,500 years old were often buried under land that experienced more recent land uses, e.g., under plaggen soils (Kooistra and Maas 2008), and were much more widespread than previously thought (Humme et al. 2013). These types of land were widespread in northwestern Central Europe (Arnoldussen 2018).

In open terrain, old structures are quite visible, but in forests, they are more difficult to detect, and a geostatistical filtering technique is needed to remove the overlying raw topography, leaving only the micro-topography visible (Humme et al. 2013). Using this technique, Arnold (2011) specifically searched for Celtic fields in ancient forests in northern Germany and remarkably discovered many large-scale Celtic fields. The findings elucidate that ancient forests were used as arable fields a long time ago and that this form of cultivation can still have an effect on the distribution patterns of plants today (see Sect. 3.1).

3.3.2 Ridges-and-Furrows

Digital terrain models have allowed the identification of former Celtic field and ridge-and-furrow structures ("Wölbäcker", Sittler et al. 2007), dating back to the Middle Ages (Trächsel 1962; Wöbse 1992; Küster 1997; Langewitz et al. 2020). It can be assumed that these arable fields were very widespread in Central Europe because many of them are still preserved under forests (Sittler 2004; Meyer 2005; Doneuses and Briese 2006).

Ridges-and-furrows (also known as "Hochäcker") are elongated, parallel strips of arable land (long strip corridors); in comparison with the furrows in between the ridges (several metres wide), the ridges are elevated in the middle, approximately 8–20 m wide and raised by up to 1 m and rarely even up to 1.2 m (Wiegand 2005; Langewitz et al. 2020). According to previous findings, these ridges-and-furrows were created by the use of a plough with a fixed spreading board. Ploughing began in the middle of the field strip, and the soil material was piled up towards the middle. A characteristic pattern of alternating ridges and deeper depressions (furrows) was created. Recent research has found evidence that the curvature could also have been caused by the addition of material, but this has not yet been sufficiently studied (but see Wiedner et al. 2017).

Turning the plough with a draught animal was an extremely laborious process that was avoided as much as possible (Küster 2010), resulting in very long strips of arable land, often several hundred metres long (Wenzel 2013). Dating these strips is not always possible with historical sources, but a recently published paper notes that the beginning of implementing this approach is often dated to the late Middle Ages (around the twelfth century), but depending on the region, earlier implementation (seventh and eighth centuries) and in a few cases even implementation in Roman times are suspected (see Langewitz et al. 2020, and the references therein). Regardless of the period of origin, it can be assumed that the structures have endured for several centuries and have survived to date in many regions (Møller 2016).

Only one study is known where ridges and furrows were investigated to determine differences in soil characteristics and effects on tree radial growth. The study showed that there were redoximorphic features in the soil in the furrows due to the existence of a less permeable soil layer, which was reflected in some stress signals in the annual rings of the related trees. On the ridges, such redoximorphic features were missing in the soil due to higher permeability and the tree rings lacked the stress signals (Hirsch et al. 2018). Another study showed that at 0–10 cm depth, the SOC content was significantly higher on average in the furrows than on the ridges, as litter accumulates (Alcántara et al. 2017). In fact, centuries after the abandonment of arable land (in this case between the seventeenth and nineteenth centuries), small-scale differences in the SOC distribution can still be detected in present-day forested areas. Notably, ridge-and-furrows are very often also preserved in ancient forests (Wulf 2001), which is a clear indication that even some of these forests with long continuity were once used for agriculture. Thus, there is the possibility that such ancient forest areas were fertilised at the time of use in the form of ridges-and-furrows, e.g., during fallow periods by grazing cattle (Wiedner et al. 2017) and applying fertiliser. However, there is as yet no evidence for this phenomenon through a study.

Considering cross-sections of ridges and furrows, one usually notices large humic horizons on the ridges and smaller humic horizons in the furrows (Schmoock and Gehrt 2017; Raab et al. 2019; Langewitz et al. 2020). These characteristics are not always clearly pronounced, as ridges and furrows can also be levelled or eroded (Schmoock and Gehrt 2017).

These findings may mean that in comparative studies of ancient and recent forests, the nutrient status may not be very different between the two types of forest, but the thickness of the upper mineral soil rich in humus may have been greater in ancient than in recent forests, but this would then not be related to habitat continuity.

3.3.3 Charcoal Burners

For a long time, charcoal was the only fuel used to reach the high temperatures required in the process of extracting iron from rocks. Charcoal burning could have been considered a sizeable business in Roman times (Marren 1990). The production of large quantities of charcoal was ensured by appropriate techniques in forestry, particularly by the charcoal burning industry. Charcoal production was local; i.e., charcoal kilns were built in the forest. The word charcoal kiln is derived from the late Latin miliarium, which means a thousand pieces and refers to the large quantity of 1–2 m long, air-dried pieces of wood, which were placed tightly around an air shaft and covered with a fireproof, almost airtight blanket of earth, leaves, moss and green grass. The pile was set alight via an air shaft filled with easily combustible material (Swieder 2019).

While the occurrence of widespread round charcoal kilns, often reaching diameters of 20 m and greater, in the European low-mountain region has been known for some time (Swieder 2019), charcoal kilns in the lowlands have only recently been reported more frequently (Raab et al. 2019). Usually, these charcoal kilns had diameters of approximately 10–12 m, which resulted in them not appearing significant as a single object in a forest. However, they can be detected in very large numbers using digital terrain models, showing that ultimately a large area within forests can be affected by these kilns (Marren 1990; Raab et al. 2019). The most important legacy is the increased amount of black carbon in the soil, which can be visually recognised in the profiles under former charcoal kilns due to the distinct darkening of the soil (Codreanu-Windauer 2019; Schneider et al. 2019; Swieder 2019).

3.4 Soils

Numerous studies on soil properties in relation to forest continuity cover a wide range of different previous uses (Table A), enabling more general conclusions on the role of legacies on soil chemistry. The majority of the studies focused on pH values, the main plant nutrients (N and P as well as Ca, K and Mg) or C accumulation and SOC stocks. In some studies, soil profile characteristics, including the organic layer, were also investigated (Table A).

3.4.1 Profile Characteristics, Including Organic Layers

Throughout the studies, the organic layers on top of the soil and the A horizon were thicker in ancient forests than in recent forests. There was one exception in a study where the L and F layers were thicker in recent forests than in ancient forests, which can be explained by pine stocking versus oak stocking in ancient forests (Dzwonko 2001a). In another case, however, the O and F layers were thicker in an ancient forest than in a recent forest (Dzwonko 2001b; von Oheimb et al. 2008). A forest restored from an area with a non-forest use shows increasing litter production, while decomposition rate is usually lower than in the open; i.e., the litter input exceeds the decomposition rate, and organic material accumulates over decades, so that adjustments in the thickness of the organic horizons from recent to ancient forests are reflected in initially lower horizon depths in the recent forests. Two studies, in which an overall thicker litter or humus layer was found in ancient forests, provide additional evidence to confirm the process of humus accumulation (Bossuyt et al. 1999b; Bergès et al. 2017).

The soil depth of areas formerly used as arable land can be deeper in recent than in ancient forests due to the use of ploughs (Abadie et al. 2018), while soil bulk density was greater in some recent forests than in ancient forests due to soil compaction with the use of machines (Wall and Hytönen 2005; Valtinat et al. 2008).

The accumulation of soil organic carbon with forest continuity is usually largely restricted to the organic layer and uppermost mineral soil horizons. It is generally visible in higher SOC concentrations in ancient forests (Petersen 1994; Wall and Hytönen 2005; Baeten et al. 2009; Bergès et al. 2017; see chapter below). In the subsoil, this effect is usually weak. In certain cases, significantly higher SOC contents have been found in recent than in ancient forests (Wall and Hytönen 2005; Vojta 2007). In one case, this pattern, which contrasts to many other observations, was explained by belowground litter from previous crops and organic amendments incorporated into the subsoil during the cultivation period, and perhaps the cool climate of the region (Wall and Hytönen 2005). In the other case, the elevated subsoil SOC was related to the previous use as a village, where considerable amounts of organic material accumulated (Vojta 2007).

3.4.2 pH Value

The pH value is almost always measured in ecological field studies, so there is a particularly high number of relevant studies available. Comparisons of ancient and recent forests generally showed higher pH values for areas previously used as non-forested sites (Vojta 2007; Bergès et al. 2017; Abadie et al. 2018; Blondeel et al. 2019). This difference existed in particular when the previous uses were settlements, gardens or arable lands, in which fertilisers and alkaline substances (i.e., ashes) have been applied. In only three studies, pH values did not differ significantly between ancient and recent forests. The recent forests in a Danish study were established 70 years ago (Graae 2000; Graae et al. 2003), which according to a study from Belgium (Bossuyt et al. 1999a) was sufficient to align the pH of recent forests with ancient forests. In this area, a drastic reduction in the topsoil pH value of former arable land occurred within 30 years after afforestation, and after only 50 years, the pH value was consistent with that of ancient forests. Only in the deeper soil layers did this process take more than 100 years (Bossuyt et al. 1999a).

This information makes it possible to interpret the findings of Hejcman et al. (2013), where the previous use of settlements dates back 600 years, with those of Wulf (2004a), where the establishment of recent forests dates back only 100 to 200 years. Wilson et al. (1997) examined very few areas but found that the variability in the pH values in ancient forests was significantly greater than that in the pH values in recent forests. This observation is noted because studies on spatial variation in soil chemistry are lacking, apart from the approaches implemented in Hornschuch and Riek (2009). However, these authors compared natural forests with commercial forests, i.e., no recent forests with explicit previous non-forest uses were included. Nevertheless, since there are so few studies, it should be noted that the authors generally found higher variability in and ranges of pH values, CEC (cation exchange capacity) and base saturation in natural forests, and these patterns also applied to the soil C- and N-contents in beech and oak stands but not in pine stands (Hornschuch and Riek 2009).

In only two studies, the pH values were higher in ancient forests than in recent forests; in one of the cases, this pattern may be explained by the pine stocking of recent forests compared to the oak stocking of ancient forests (Dzwonko 2001b). In the other case, only two ancient and four recent stands were compared (Baeten et al. 2009), so the result should be evaluated against this small sample size.

3.4.3 Mineral Soil C, N and P Content

SOC contents were generally higher in ancient forests than in recent forests. The exceptions were related to former intensive human uses such as settlements or gardens (Table A) that had high inputs of C-containing substances (e.g., building rubble, fertilisers and food waste). These findings are supported by two studies (Thuille et al. 2000; Thuille and Schulze 2006), whose chronosequences do not go beyond 130 years and do not compare ancient with recent forests but complement the picture very well. Thuille and Schulze (2006) found the highest amount of total C stock in the oldest forest stands in a secondary succession series from 0 (grassland) to 122 years and determined that it took at least 80 years to reach the SOC stock prior to clear-cut in the mineral soil again. These results are comparable to those of Vesterdahl et al. (2002) because the authors found the highest C storage in approximately 200-year-old oak stands compared to almost 30-year-old arable afforestation. Additionally, Thuille et al. (2000) found an increase in the C stock in the soil organic layer with increasing stand age over a chronosequence from 0 (grassland) to

62- and 130-year-old coniferous stands. After 60 to 80 years, C accumulation in the wood biomass decreased but continued to increase in the soil organic layer.

In the case of soil nitrogen content, the findings for higher values in ancient or recent forests almost balanced each other (Table A). However, $\delta^{15}N$ signatures in soil material reached higher values in recent forests than in ancient forests, regardless of whether these forests were previously used as fields, grasslands, gardens or settlement areas (Koerner et al. 1997, 1999), which might indicate former input of mineral N fertiliser. The net nitrate production was also higher in formerly manured soils than in ancient forests (Jussy et al. 2002). The N_t content was higher in the Ap horizon of a 1- and 29-year-old Norway spruce plantation than in an approximately 200-year-old mixed deciduous forest (Ritter et al. 2003). One study provided a different picture of N_t contents because they were higher in the O-horizon in ancient forests but higher at depths of 0–10 and 10–20 cm in recent forests (Wall and Hytönen 2005).

The C/N ratio was usually higher in ancient forests than in recent forests (Blondeel et al. 2019), suggesting more rapid accumulation of C than N with increasing forest age. The C/N ratio was higher in recent forests than in ancient forests in only one study (Janssen et al. 2018), which was not explained by the authors and could not be related to specific conditions of the study plots. A possible explanation might be assumed former litter removal. Litter raking, which has been known to occur since at least the Middle Ages (Leuschner and Ellenberg 2017), was widespread and peaked in Europe in the middle of the nineteenth century, with approximately 50 Tg of dry litter per day taken (McGrath et al. 2015). Several experiments on litter use have demonstrated that litter raking removes large amounts of N, P and basic cations (Onno 1969; Kreutzer 1972; Dzwonko and Gawronski 2002). In Switzerland, it has been shown that after 310 years, this use led to an average reduction in SOC of 17%, and that 130 years after abandonment of this use, legacy effects are still visible. The formerly litter-raked forested areas are currently acting as C sinks, as carbon re-accumulates (Gimmi et al. 2013).

Various studies have measured available P and total P in the soil of ancient and recent forests. Available P was in most cases higher in recent forests than in ancient forests; an exception was the study of Wulf (2004a) with no significant difference. For total P, the number of findings of higher values in ancient and recent forests balanced each other. Wall and Hytönen (2005) showed that in the topsoil (0–10 cm), the P values in recent forests were higher, while in deeper soil layers (30–40 cm), the P values in ancient forests were higher. A higher C/P ratio in ancient forests than in recent forests was only documented in one study (von Oheimb et al. 2008), which contrasts with the situation for soil C/N ratio.

High P contents in the soils of former Roman settlements were also reflected in the leaves of oaks that grow on such soils (Dupouey et al. 2002). These findings are supported by a study from Belgium (Baeten et al. 2009), in which the tenfold increase in available P in the soil was reflected in elevated biomass P contents.

Different findings for the C, N and P values can, as already indicated above for P, be best explained by (1) measurements in soil horizons of different depths or inclusion/non-inclusion of the organic layer, (2) differences in tree species, as the root systems may play an important role in C accumulation at greater depths, (3) different former land uses and (4) habitat continuities of different lengths. Leuschner et al. (2014) found significantly higher C and N pools in the mineral soil (up to approximately 30 cm) but not the organic layer of ancient (>230 years continuity) compared to recent beech forests (50–128 years continuity). Decades or centuries of former land uses have apparently reduced the C and nutrient storage of the subsoil. However, the soil that Leuschner et al. (2014) referred to as subsoil was referred to as topsoil (up to 28 cm depth) by Nitsch et al. (2018). A study on three tree species (beech, oak and pine) clearly showed that in comparison with old forests (100–200 years continuity), ancient forests (>230 years continuity) stored significantly more SOC, N and P, but for SOC, this was true only in the subsoil (29–55 cm) and not in the topsoil (0–28 cm) (Nitsch et al. 2018). The former land uses of the investigated recent or old forests were relatively comparable in both studies noted above (grassland or fallow land, and in the study of Leuschner et al. 2014 very little arable land). The main difference between the studies lies in the duration of habitat continuity, which may explain the different findings. Finally, tree species also played an important role because Nitsch et al. (2018) found 50% larger SOC stocks on average in ancient beech and pine stands than in ancient and old oak stands (each considered for a soil depth of 29 to 55 cm). Differences between beech and pine trees existed in this study, but they only became clear, when looking at different soil horizons. Pine afforestation resulted in higher C stocks in the organic layer (Leuschner et al. 2013) or in the upper mineral soil to approximately 40 cm depth (Nitsch et al. 2018). On the other hand, beech stands had higher C stocks in the mineral soil (Leuschner et al. 2013), or in the subsoil at depths between 40 and 55 cm (Nitsch et al. 2018). These findings highlight that a distinction must be made between tree species (but see Ritter et al. 2003) and horizons, and that a number of research questions still remain unanswered.

3.4.4 Mineral Soil Ca, K and Mg Content

Calcium, potassium and magnesium usually occurred at higher concentrations in the soil of ancient forests than in recent forests (Table A). The only exception was found in the study by Hejcman et al. (2013), which refers to areas with a former settlement and shows that recent forest stands were still enriched with Ca, K and Mg after 600 years. In some cases, the findings on these elements have been relatively detailed, such as those of Wall and Hytönen (2005), where K in only the O-horizon and Mg in only the 0–10 cm depth of the mineral soil had higher values in the ancient forest than in the recent forest, while the same applied to Ca for the O-horizon and 0–10 and 10–20 cm soil depth. In the study of Wilson et al. (1997), the concentrations of these elements were 50% higher in the ancient forests, but the differences to the recent forests were not significant. In addition to this finding, the CEC in the A_{eh} horizon or topsoil was higher in ancient forests than in recent forests, as shown by studies from Poland and Germany (Dzwonko 2001b; von Oheimb et al. 2008).

3.4.5 Soil Biological Activity and Mycorrhizal Associations

Only one study from Germany investigated the microbial biomass and enzymatic activity in the soil of recent forests compared to soil of ancient oak forests. It showed higher microbial activities and a higher abundance of AM mycorrhizal fungi in the recent stands (Fichtner et al. 2014), which relates to higher N and P contents in the soil. Humphrey et al. (2004) investigated forests on former heathland and grassland and found more forest-typical mycorrhizae in those stands that were closer to ancient forest stands.

Recent studies from Belgium showed that in the early-successional woody species alder (*Alnus glutinosa* (L.) Gaertn.) and hawthorn (*Crataegus monogyna* Jacq.), there were no clear effects of forest continuity on ectomycorrhizal communities, but soil conditions affected these communities. However, forest continuity had an effect on the ectomycorrhizal communities of hazel (*Corylus avellana* L.), which is typically associated with ancient forests (Boeraeve et al. 2018a). Furthermore, ectomycorrhizal communities in recent forest stands adjacent to ancient stands were more similar than those found in isolated recent stands, and isolated recent stands had a significantly lower diversity of ectomycorrhizal fungi and were colonised mainly by highly dispersive species (Boeraeve et al. 2018b).

3.4.6 Soil Moisture

There is a lack of comparative studies between ancient and recent forests regarding soil moisture conditions or ecosystem water fluxes. Only Valtinat et al. (2008) carried out such a study and found that the moisture in the upper soil (0–5 and 5–15 cm) was higher in ancient forests than in recent forests over all four seasons.

3.5 Biomass and Productivity of Forests

Three studies found that trees growing in ancient forests are less sensitive to climatic extremes than trees growing in recent forests (von Oheimb et al. 2014; Mausolf et al. 2018; Mausolf et al. 2020). In Spain, beech trees that established on former arable and pasture lands more than 50–60 years ago showed a 32% higher growth rate and a 3% lower wood density than those in ancient forests, and this difference was still significant, when controlling for differences in tree age and competition intensity. In the study by Mausolf et al. (2018), higher specific fine root length and specific root area and lower root tissue density were observed in the beech trees in the recent compared to the ancient forests; on the other hand, fine root system size was larger in the ancient forests. These observations indicate that climate-growth relationships in European beech may critically depend on former land use, suggesting that ancient beech forests may be less susceptible to climate extremes. A larger fine root system and apparently more robust fine roots may play a decisive role for this difference.

Von Oheimb et al. (2014) found that sessile oaks (*Q. petraea* L.) growing on former arable land had higher radial growth rates and showed a higher climate sensitivity of growth than oaks in ancient forests. The higher growth rates were related to higher soil N and P contents due to previous fertilisation. Not only woody species benefit from the increased nutrients in recent forest soils after previous agricultural use; six of seven herbaceous species studied showed significantly higher biomass production as well (Baeten et al. 2011). In forested areas formerly used as coppice-with-standards, Altmann et al. (2013) observed that oaks showed stronger growth after coppice intervention, i.e., when more light was available for the oaks due to the drastic pruning of the coexisting lime trees.

4 Synthesis and Further Research

The 181 reviewed publications showed that previous non-forest uses can still be relevant to the occurrence of certain plant, animal and fungal species or to the species assemblages decades to many centuries later. Such legacies are generally more evident on lands that experienced more intensive use and/or longer durations of non-forest uses. This pattern can be seen in the legacies of topographical features (see Sect. 3.3), which are detectable by LiDAR in large parts of central Europe (e.g. Sittler et al. 2007). The use as arable land or grassland, often only a few decades ago, is reflected in the presence or absence of certain indicator organisms. In ancient forests, typical forest organisms occur more frequently, whereas in recent forests (post-agricultural forests), typical non-forest organisms, i.e., organisms that also occur in open areas, are noticeably more frequent. The plants, animals and fungi that occur exclusively or significantly more frequently in ancient forests essentially have in common that they are not, or are only insufficiently, adapted to long-distance dispersal. Given this background and the strong fragmentation and the associated decline in area of ancient forests, the latter have been assessed as habitats of high conservation priority, particularly in Central Europe (Goldberg et al. 2007; Bradshaw et al. 2015; Wulf 2018). Their conservation value undoubtedly also lies in the fact that among certain organisms primarily restricted to ancient forests, several are rare or listed as endangered species, and ancient forests function as source areas for the colonisation of newly created or future habitats. Recent forests should be seen as not only potential new habitats for such organisms, but also as "landscape archives" of human land use history.

All studies to date point directly or indirectly to the need to consider legacies in order to gain a more complete understanding of present-day patterns and processes in ecosystems or landscapes. The thousands of years of human use have been exhaustively described for Central Europe's forested landscapes (e.g. Leuschner and Ellenberg 2017; Poschlod 2017), and it is known that by approximately 1600 AD, there was no longer any unmanaged forested area in this region (McGrath et al.

2015). Moreover, the millennia of human impact have decimated and fragmented the forest area and have largely changed tree species composition towards conifer plantations. The remaining forests bear a large-scale "memory of the landscape". This situation results in a number of challenging tasks for various fields of research (Bergès and Dupouey 2021). From this review, the following key research questions arose, which have not been answered or have been answered only incompletely.

Research question 1: How long remains the ecological memory of the various former non-forest uses?

Future studies should focus on the following three areas, when addressing this research question: (i) the effects of legacy types that have not been studied so far; (ii) the effects of ridges and furrows in forests, which have rarely been studied, and (iii) regional differences in legacies. Regarding (i), the numerous abandoned villages with associated farmland that are now covered by forest should be studied in detail (e.g., Otte 1996). Regarding point (ii), it should be considered that ridges and furrows, which are often also recognisable in ancient forests, can still have an effect on tree growth today, if former use included fertilisation. Systematic studies on this aspect are completely lacking. Finally, more information on regional differences in land-use legacies in the forests of Central Europe is needed (focus iii) (cf. Bergès and Dupouey 2021).

Research question 2: How are changes in ecosystem state factors and processes due to past land uses modulated by current environmental changes (e.g., climate change and N deposition)?

Elevated P levels from past agricultural use can increase the reaction of plant communities to elevated N levels or significantly attenuate their response at low P levels (Marrs 1993; Ollinger et al. 2002). Such interactions between past land uses and current N deposition as well as other current environmental changes illustrate that further changes in ecosystems are modulated by previous environmental changes. Perring et al. (2016) highlight that the potential of this modulation has been overlooked until now. They support their statement with an extensive literature review and therefore argue that future research should target the interactions between legacies and multiple environmental changes (see also Question 39 in Ammer et al. 2018).

Research question 3: What are the consequences for regional and global modelling of ecosystem processes such as C sequestration, if a distinction is made between ancient and recent forests and between different former uses?

The slow accumulation and long residence time of carbon in biomass and soil explains the slower adaptation rate of biogeochemical fluxes in comparison with community composition in recent compared to ancient forests (Vesterdahl et al. 2002; McGrath et al. 2015). The re-accumulation of C and nutrient elements in forests after over-exploitation may last several hundred or even thousands of years (McGrath et al. 2015; Dupouey et al. 2002; Falkengren-Grerup et al. 2006; Hartshorn et al. 2006). These findings have far-reaching consequences for the current assessments of C sequestration at larger spatial scales and for predictions on the future C sink in forested areas. Based on high-resolution time series of anthropogenic deforestation in Europe over the last three millennia, Kaplan et al.

(2009) find that humans have disrupted the carbon cycle as early as 1000 BC to a higher degree than hitherto thought and that early effects on the climate system may have been greatly underestimated. There is also evidence that future C sequestration in forests is dependent on previous uses or abandonment of historical uses (Laganière et al. 2010; Thom et al. 2018). A main conclusion is that neglecting legacies can lead to distortions in the assessment of future processes in forests (Thom et al. 2018).

Research question 4: What role did humans play in the distribution and establishment of certain organisms in the context of historical farming?

All approaches to explain today's distribution patterns of species (trait-based analyses and calculated annual dispersal distances, see Sect. 3.1) cannot, for example, conclusively explain the overall distribution pattern of *Anemone nemorosa* L. in the southern Swedish landscape over hundreds of km after glacial retreat (Brunet and von Oheimb 1998). Therefore, the well-known Reid's paradox (Clark et al. 1998) remains unexplained. Even studies on wild animals as potential vectors for the dispersal of diaspores over longer distances do not provide a satisfactory answer either, as they show that mainly non-forest species were found on the coats and hooves or in faeces of large and small game animals (Heinken et al. 2002; Heinken and Raudnitschka 2002; Schmidt et al. 2004). Moreover, the absolute numbers of diaspores found in the coats of roe deer and wild boar were quite low (well below 10, often only one or two seeds that have germinated; Schmidt et al. 2004), and effective dispersal to establish populations over large areas was unlikely. In fact, answers are still lacking regarding how typical forest plant species spread and established populations over large landscapes after the retreat of glaciers. It is not yet clear which vectors were effective in this process.

Research question 5: Why do certain plant species differ in their association with ancient forests in different regions?

To date, the same species in one European region can have a very strong association with an ancient forest, but not have the association in another region. For example, *Melica uniflora* Retz. and *Mercurialis perennis* L. are considered indicator species of ancient forests in several European countries but not in Denmark (Hermy et al. 1999). Perhaps, differences in the extent of former forest cover, inclusive connectivity of forested areas and the duration of connectivity of forested areas are influential. It is interesting that for explaining the presence and species richness in endangered taxa, the forest cover 200 years ago has a higher predictive power than the current forest cover (Flensted et al. 2016).

Research question 6: Are there further animal species and, in particular, soil organisms that indicate legacies of former non-forest uses?

Although there is a relatively large number of publications that list animals as ancient forest indicator species (e.g., Harding and Rose 1986; Alexander 1996), the number of comparative studies with recent forests is quite small. Nevertheless, Nordén et al. (2014) showed in a review study that habitat continuity at the local level is important for certain species of insects and land snails, and continuity at the landscape level has an effect on the current occurrence of certain invertebrate species. The authors concluded that many species in landscapes with fragmented forest areas are limited in their distribution and probably mainly occur in landscapes with ancient forests, because the availability of habitats in ancient forests appears to have been greater in the past than today. However, apart from few studies such as Bowen et al. (2007) on the recovery of the fauna in regrowing forests, there is a general lack of systematic investigations on the role of legacies for the fauna of forests.

5 Conclusion

Ecosystems and landscapes have an ecological memory, which is expressed in abiotic and biotic legacies (Jögiste et al. 2017) that can be traced for decades, centuries or even millennia (De Frenne et al. 2011). The longer and/or more intensive the former human impact, the stronger and longer lasting the memory effect; i.e., the more clearly legacies can be recognised or proven. Nevertheless, there are examples of only minor human activity in the past that have led to long-lasting legacies (e.g., Freschet et al. 2014). Appropriate periods of time must be considered when identifying or proving legacies (Essl et al. 2015). One of the most common problems in researching legacies are limitations in the quality and quantity of historical information sources on the duration and intensity of former uses (Bürgi et al. 2017). However, there are some European countries that have presented comprehensive inventories of ancient forests based on various historical sources, for example, England, Wales (Spencer and Kirby 1992; Sansum and Bannister 2018), Ireland (Perrin and Daly 2010) and Germany (Glaser and Hauke 2004). Historical ecology is a promising field of research for addressing open questions on the functioning of forest ecosystems, provided that the various involved disciplines agree on a uniform methodology and find the way to joint research agendas (Szabó 2015; Armstrong et al. 2017).

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Appendix

Data availability. The compiled data on which this study is based are presented in Table A. Detailed primary data are stored and published in the BonaRes repository and are available online at: https://doi.org/10.4228/zalf.2ftb-s553.

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Global Forest Biodiversity: Current State, Trends, and Threats

C. Leuschner and J. Homeier

Contents

Abstract Human activities, especially forest conversion and degradation, are causing global declines in forest biodiversity. This review quantifies the current extent of the major forest biomes on earth and their area losses in historical and recent time. The importance of global forests for the earth's terrestrial biodiversity is explored and the role of forest degradation, fragmentation, defaunation, and forest fires for

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forest biodiversity analyzed based on the comparison of managed and unmanaged forests and reported forest biodiversity trends. The outstanding role of the remaining primary forests for global forest biodiversity is highlighted, the imprint of millenia of forest use on forest biodiversity explored using Germany's forests as an example, and a brief assessment of the impact of climate change on forest biodiversity given. We conclude that conserving the last remaining primary forests is of paramount importance for the future of biodiversity on earth. When a substantial part of the earth's forest-related biodiversity is to be inherited to future generations, a global effort to establish at least ten effectively managed forest mega-reserves in the tropical and boreal forest biomes is urgently needed.

Keywords Biodiversity trends, Boreal forest, Defaunation, Forest degradation, Forest fragmentation, Forest loss, Forest management, Species numbers, Temperate forest, Tropical forest

1 Review Scope

Forests are of paramount importance for the future of biodiversity on earth. This is particularly true for tropical forests with their enormous species richness, but also for the other forest biomes, which are less species-rich, but are home to a large number of specialist taxa. This review attempts to summarize our knowledge about the current state of biodiversity in the forests on earth. The focus is on plant life, but the status of forest-dwelling animals and fungi is also addressed. Much attention is paid to deforestation and the different forms of forest degradation and their impact on forest biota. Even though the proportion of secondary forests in the global forest stock is continuously increasing, and secondary forests should therefore unquestionably be included in conservation concepts, we focus in this review on primary forests and their degradation stages due to their outstanding importance for the characteristic biodiversity of forests.

2 Definitions of Forest and Forest-Use Categories

Forests are defined very differently worldwide (Lund 2018). With regard to biodiversity, it makes sense to differentiate between closed forest (canopy closure >70%), open forest (40–70%), forest landscapes (20–40%, i.e., mosaics of forest and treeless ecosystems), and tree savannah (5–20%). The FAO Forest Resources Assessment (FRA) uses somewhat different definitions that lead to varying estimates of forest extent: closed forest (>40%) and open forest (10–40%). In forest censuses based on the FRA, all woodlands with >10% canopy closure are counted as forests, whereby the area colonizable by species bound to closed forests is significantly overestimated (Chazdon et al. 2016). With regard to forest use, the following categories are distinguished (Putz and Redford 2010): (1) degraded forest (primary forest, which has lost its characteristic structure and species composition due to human use), (2) managed forest (forest that is purposedly domesticated for production of forest products), (3) secondary forest (forest that grew naturally after complete forest destruction), and (4) plantations (stands resulting from tree planting or artificial seeding).

3 Original Forest Extent

Approximately 47% of the earth's land surface was covered by forest 8,000 years ago before humans began clearing large areas. This corresponds to an estimated total area of 62 million km^2 (Billington et al. 1996). In the following, the four most important forest biomes in terms of area are looked at: The tropical forests (with moist and dry forests), the subtropical forests (with ever-moist subtropical laurel forests and summer-dry subtropical, sclerophyllous hardwood forests), the temperate forests (with nemoral deciduous, mixed and coniferous forests), and the boreal forests.

Tropical moist forests are believed to have originally existed on approximately 16–17 million km^2, temperate and boreal forests each on 15–16 million km^2 (Kuusela 1992; Schultz 1995; Wright 2010; Martin 2015). For the subregions of the tropical moist forest, Asner et al. (2009) give original areas of 8.83 (South America), 0.69 (Central America and the Caribbean), 2.92 (Africa), and 7.19 million km^2 (Asia and Oceania); that is slightly more than mentioned above (total: 19.62 million km^2). The natural extent of the subtropical forest biome was significantly smaller (approx. 8.8 million km^2). The global extent of dry forests (>40% canopy closure) was estimated at 7.77 million km^2 in 2015 (Bastin et al. 2017), with most of the area in tropical and subtropical regions.

Already millenia ago, man has begun to transform the temperate forests of East Asia (in China since the second millennium BC) and Europe (since the Neolithic and increasingly from the early Middle Ages onwards) into agricultural land, excepting only small parts. The large-scale transformation of the subtropical hardwood forests of the Mediterranean region started in Greek and Roman times. In the temperate forests of North America, large-scale forest destruction has happened only in the course of European settlement since 1,500. This long-ago destruction of temperate and Mediterranean forests was undoubtedly associated with biodiversity losses, which can be at least partially reconstructed through biodiversity inventories in the few remaining primary forest areas. However, large parts of the forest-dwelling herbivorous and carnivorous megafauna in temperate, tropical, and boreal forests have been decimated by hunters already in early prehistoric times, with consequences for forest structure, food webs, and biogeochemical cycles (Malhi et al. 2016; Galetti et al. 2018). It has to be kept in mind that most forests of the world have been subject to more or less intense human influence since several thousands of years, often altering forest structure and species composition in the distant past, long before forest destruction started (Ellis et al. 2021).

In contrast, large-scale forest loss in the tropical moist and dry forests has only occurred in the last five decades. This is also valid for the subtropical forests outside of the Mediterranean region, the southern hemispheric temperate forests, and the boreal forests.

4 Recent Forest Area and Deforestation Rates

4.1 Forest Area

More or less systematic surveys of global forest area and its changes have been carried out only since the 1980s and 1990s with the advent of remote sensing technology and the national forest inventories stimulated by the FAO (Forest Resources Assessments; FRA 2020). Despite groundbreaking advances in the quality of satellite data, there are still inconsistencies in the determined areas of different forest categories and their cover changes over time, which make it difficult to estimate primary forest losses. The causes are a lack of uniform standards with regard to the differentiation of forest and tree-covered open land, the often insufficient distinction between primary forest, secondary forest and tree plantations, the handling of forest fragmentation and the smallest forest patches, as well as constant cloud cover in some regions (FRA 2020; Global Forest Watch 2021).

The FAO census gives for 2020 a global forest area of 40.6 million km^2 (i.e., 31% of the global land area), with 10.09 million km^2 in the boreal, 6.65 million km^2 in the temperate, 4.49 million km^2 in the subtropical, and 18.34 million km^2 in the tropical forest biome (including dry forest) (FRA 2020; Fig. 1). According to the FRA classification, 45% of the remaining forest area in 2020 was tropical forest, 27% boreal forest, 16% temperate forest, and 11% subtropical forest (Fig. 2). Considering only the tropical moist forest, the area was estimated in 2012 to 10.95 million km^2 according to high-resolution satellite images (Hansen et al. 2013). Mangrove forests covered 148,000 km^2 in 2020 (FRA 2020).

4.2 Forest Loss

The net forest loss is estimated by the FAO at around 4.2 million km^2 in the period 1990–2020, with more than 90% of it having occurred in tropical forests (FRA 2020). Global forest loss has decreased by a third from on average 158,000 km^2 per year in 1990–2000 to 102,000 km^2 per year in 2015–2020 (and from 138.000 to 92,800 km^2 year^{-1} in the tropics alone) (Fig. 3).

Recent forest loss is by far greatest in the tropical biome. Compared to the assumed original area of around 16 million km^2 (Martin 2015), it is estimated that around 28% of the tropical moist forest area was already lost before 1990: Four

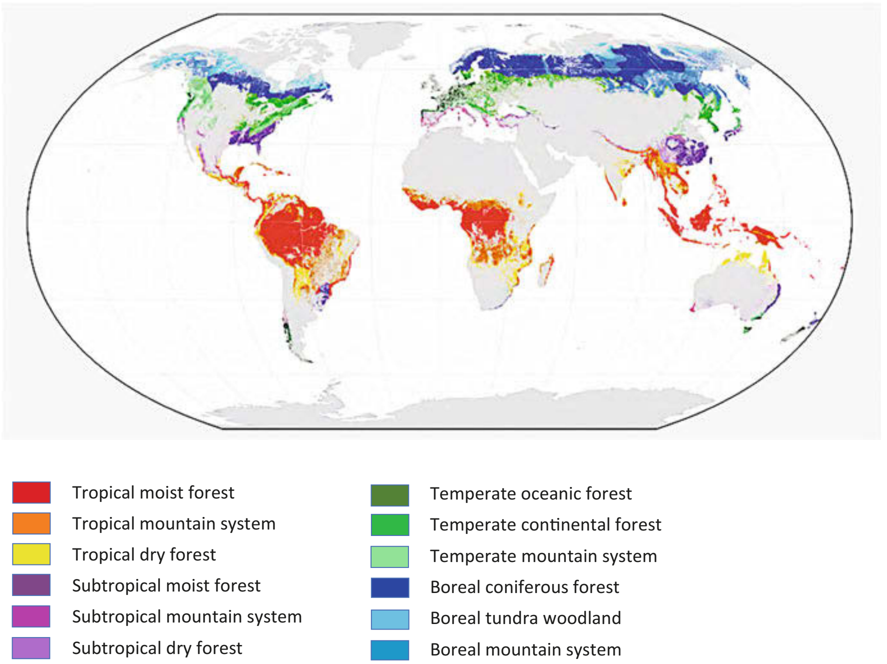

Fig. 1 Distribution of the four major forest biomes (tree cover >30%) with subtypes in 2015 according to Copernicus moderate-resolution (100 m) land cover map. Source: FAO based on Buchhorn et al. (2020)

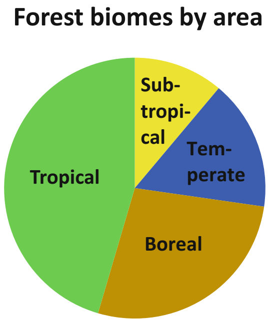

Fig. 2 Global extent of the four main forest biomes in 2020. The tropical biome includes tropical moist and dry forests. Prepared by FAO based on FAO global ecological zone map (FAO 2012) and global Copernicus Land Cover Map for 2015 (Buchhorn et al. 2020)

different surveys estimated the tropical moist forest area in the early 1990s to 11.50–11.72 million km^2; the numbers for the continents differed more widely (Latin America: 6.52–6.93 million km^2; Africa: 1.98–2.18 million km^2; South and Southeast Asia/Oceania: 2.71–3.02 million km^2). An evaluation of the Hansen et al. (2013) data by Martin (2015) gave an annual gross loss of tropical moist forest area

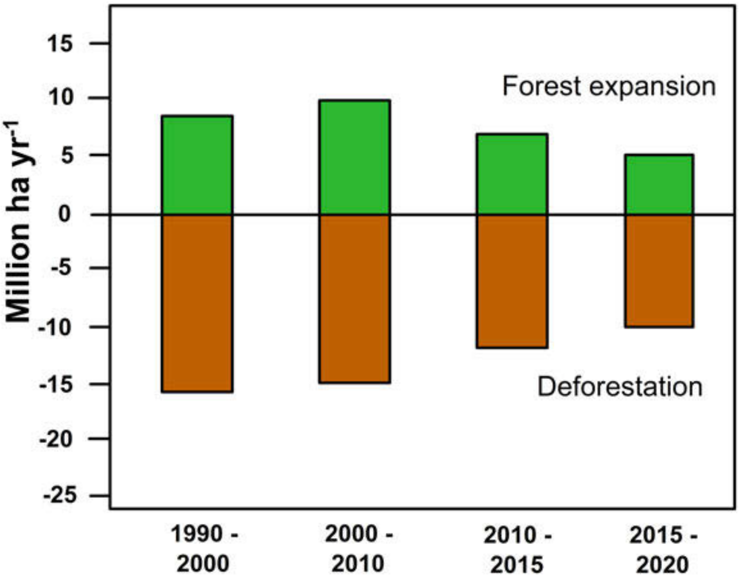

Fig. 3 Global deforestation and forest expansion between 1990 and 2020. After FAO and UNEP 2020

of 49,000 km^2 (0.43% per year) for forests with more than 50% canopy cover in the years 2000–2012. If the area gains through reforestation or afforestation of 0.08% per year (14,000 km^2) are included in the calculation, the net loss accounts to 0.31% per year. The three tropical forest regions differ significantly in their deforestation trends: In Brazil, deforestation peaked in 2004 and then declined as a result of the government's action plan to curb deforestation (Silva Jr et al. 2020). Since then it has varied between <10,000 and 20,000 km^2 per year (Curtis et al. 2018) and amounted to 182–187,000 km^2 in the period 2001–2013 (Tyukavina et al. 2017), with a recent upward trend in 2019 and 2020 (Silva Jr et al. 2020). Deforestation in the Atlantic rainforest region of Brazil has been particularly dramatic. It originally covered an area of 1.5 million km^2 and was reduced to 164,000 km^2 (11.7%) today with only small fragments remaining (Ribeiro et al. 2009). The rate of deforestation in Southeast Asia, particularly in Indonesia, rose continuously in the period 2000–2016 to more than 7,500 km^2 per year. It includes large primary forest losses (95,000 km^2 in the period 2002–2019; Global Forest Watch 2021), to a considerable extent also in protected areas (Curran et al. 2004; Margono et al. 2014). Since 2017, rates have decreased in Malaysia and Indonesia due to legal action. In the Congo Basin, there was a forest loss of 166,000 km^2 (most of it in the Democratic Republic of Congo) in the period 2000–2014 with an increasing trend (Tyukavina et al. 2018); 44% of them were primary and old secondary forests (compare also the forest loss data provided continuously by World Resources Institute: www.wri.org/initiatives/global-forest-watch and https://research.wri.org/gfr/global-forest-review).

5 Biodiversity in Forests

Forests are crucial for the conservation of biodiversity. Projections assume that 50–80% of all terrestrial organism species live in forests, which is considerably exceeding the global area share of the forest (Wilson 1988; FAO and UNEP 2020). However, biodiversity is unequally distributed, with major differences among and within the forest regions of the world.

About two thirds of the species described so far (approx. 1.9 million taxa, Régnier et al. 2015) occur in the tropics, most of them in tropical moist forests (Pimm and Raven 2000; Groombridge and Jenkins 2003). In fact, many organism groups reach their highest diversity in tropical forests. About two thirds of all land plant species are native to the tropics, probably more than 250,000 species (Antonelli and Sanmartín 2011; Pimm et al. 2014; Pimm and Joppa 2015). All five global megadiversity centers with >5,000 vascular plant species on 10,000 km^2 are located in tropical forest areas (Barthlott et al. 2005). About 20% of all tropical plant species are trees (~ 50,000 species, Fine et al. 2008; Beech et al. 2017), of which 19,000 to 25,000 each occur in Latin America and Southeast Asia, while the African tropical forest is home to only 4,500 to 6,000 tree species (Slik et al. 2015). The tree species numbers in the temperate (~1700) and boreal zone (~160; Fine et al. 2008) are by magnitudes lower.

At the local scale, almost as many tree species may exist in a single tropical forest patch as are occurring in all temperate forests of the world combined (Wright 2002). This is demonstrated by the larger tropical lowland plots of Lambir in Sarawak (52 ha: 1175 tree species ≥1 cm BHD) and Yasuni in Ecuador (25 ha: 1104 species). Certain 1 ha-plots in the tropical moist forest can harbor more than 300 tree species with diameters >10 cm (Valencia et al. 1994: Ecuador). Vascular plant species richness can be enormous even on small areas (0.01 ha: 233 species, Whitmore et al. 1985; 0.1 ha: 300–400 species, Duivenvoorden 1994; Galeano et al. 1998; 1 ha: 942 species, Balslev et al. 1998).

Most tropical tree species are locally rare and have small distribution ranges, but some are widespread and dominant. For example, ter Steege et al. (2013) estimated that of the approximately 16,000 tree species in the Amazon, 227 can be considered hyper-dominant and account for half of all tree trunks in the region. Another 11,000 species are so rare that together they make up only 0.12% of the stems. Nearly 58% of all tree species on earth are single-country endemics (Beech et al. 2017), many of them in tropical forests.

Most of the more than 31,000 known vascular epiphytes are found in tropical moist forests. This life form represents about 10% of all vascular plants on earth (Zotz and Bader 2009; Zotz et al. 2021).

It is estimated that tropical and subtropical moist forests are home to around 20,000 vertebrate species (mammals, birds, reptiles, amphibians), tropical and subtropical dry forests to about 7,000 species, temperate deciduous and mixed forests to 4,500 species and boreal forests to 1,000 species (MEA 2005). Global diversity centers are located almost exclusively in the tropical forests (Jenkins et al.

2013). The Amazon, southeastern Brazil and Central Africa together house about 50% of all vertebrate species. Almost 70% of all bird species (6,900 out of >10,000) occur in forests (Unwin 2012). Of the 6,093 tropical bird species, 53% are forest specialists and are therefore bound to forests (Sekercioglu 2012). More than 80% of the world's amphibian species are found in forests, the majority of which occur in tropical forests (Stuart et al. 2004).

While much less is known about invertebrate taxa, it appears that more than half of the estimated six to eight million terrestrial arthropod species occur in tropical moist forests (MEA 2005; Hamilton et al. 2013). In a protected area in Panama, Basset et al. (2012) detected 6,144 different arthropod species on just 0.48 ha of rainforest. They estimate the total arthropod diversity in the 6,000-ha San Lorenzo Reserve at 25,000 species. However, high arthropod species densities have also been found in near-natural temperate forests, such as 5,000–6,000 arthropod species on 60 ha of protected beech forest in Central Germany (Dorow et al. 2010).

6 Primary Forests and Their Biodiversity

Because of its outstanding importance for biodiversity, the area of intact primary forest deserves special attention (Gibson et al. 2011; Watson et al. 2018). Forests that remained largely untouched by human activities are more diverse in most organism groups than managed or degraded forests and especially agricultural transformation systems (Barnes et al. 2017). This probably also applies to the number of not yet identified species (Giam et al. 2012). Based on the information provided by the states, which, however, use non-uniform definitions, the FRA specifies a global primary forest area of 11.1 million km^2 for 2020, around 28% of the total forest area in the FRA listing (FRA 2020; see also Morales-Hidalgo et al. 2015). According to these data, more than 810,000 km^2 of primary forest have been lost between 1990 and 2020 globally, but precise data for Russia are missing (most likely >500,000 km^2 alone). According to the FRA, the global primary forest loss rate has decreased from 34,100 km^2 per year in 1990–2000 to 12,700 km^2 per year in 2010–2020 (FRA 2020); other inventories produced higher loss rates. The World Resources Institute (2020) gives average global loss rates of tropical primary forest of about 30,000 km^2 year^{-1} for the period 2002–2019, with higher figures in 2016 and 2017 (Fig. 4). Recent primary forest losses were highest in Brazil (20,000 km^2 per year in 2000–2010), in the Democratic Republic of Congo (7,230 km^2 year^{-1} in 2010–2020) and in Indonesia (7,130 km^2 year^{-1} in 2000–2010 with lower figures thereafter). An inventory based on satellite image analysis gave for 2013 an area of intact large-scale forest landscapes without human activities of 11.89 million km^2, of which about 5.6 million km^2 were in the tropical and five million km^2 in the boreal forest biome (Potapov et al. 2017). This area shrunk by 7.2% from 2000 to 2013.

The concepts of "frontier forests," "intact forest" (Watson et al. 2018) or "intact forest landscapes" (i.e., forest mosaics $\geq$500 km^2 dominated by primary forest; Potapov et al. 2017) or "hinterland forests" (i.e., late-successional tropical forests

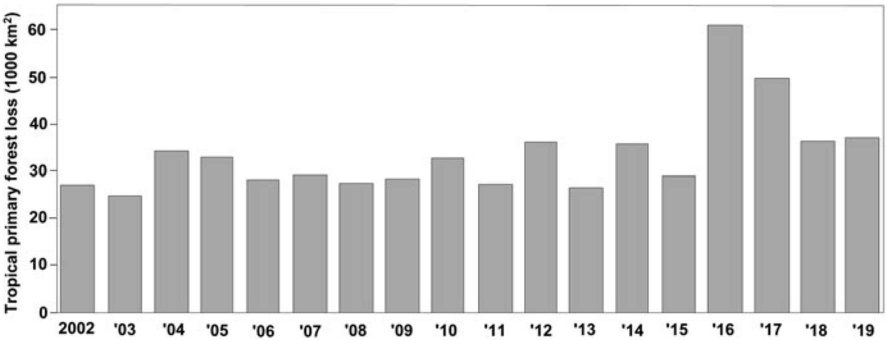

Fig. 4 Annual global loss of tropical primary forest from 2002 to 2019. After World Resources Institute (2020)

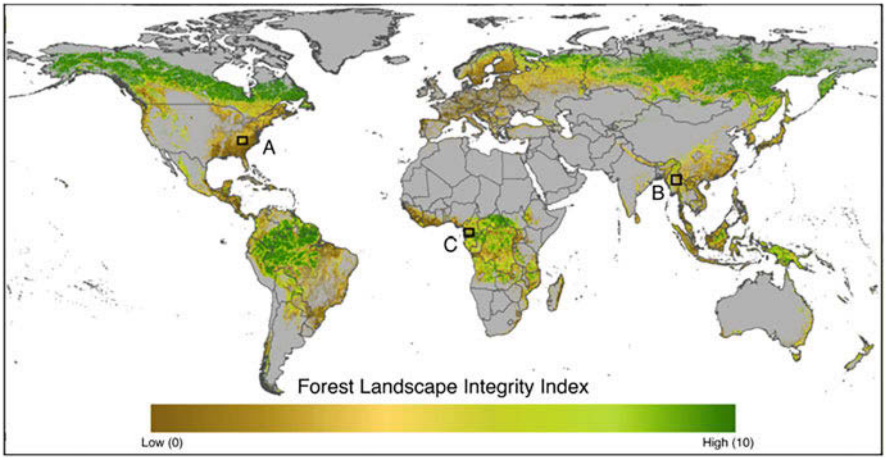

Fig. 5 Global map of forest integrity for the start of 2019. After Grantham et al. (2020). In the regions A, B, and C, case studied were conducted by the authors

$\geq$100 km^2; Tyukavina et al. 2016) consider forests that are largely uninfluenced by humans, are unfragmented and of sufficient size to maintain large-scale natural forest dynamics (Mackey et al. 2020). According to Bryant et al. (1997), in 1995 22% of the original forest area worldwide was preserved in a form that qualified it as frontier forest. This is less than the primary forest area estimated in the FRA 2020 25 years later, demonstrating inconsistent definitions. In fact, the global distribution of intact forest area can be visualized by means of different metrics (Watson et al. 2018). Here, the maps of forest landscape integrity of Grantham et al. (2020) (Fig. 5) and of forest biodiversity intactness of Hill et al. (2019) (Fig. 6) are displayed. Half of the intact forest area was in the boreal forest, only 3% in the temperate forest, and around 40% in the tropical forest. Seventy-five percent of the remaining primary forest area was constituted by three large forest blocks, i.e. in the boreal forest zones of Russia,

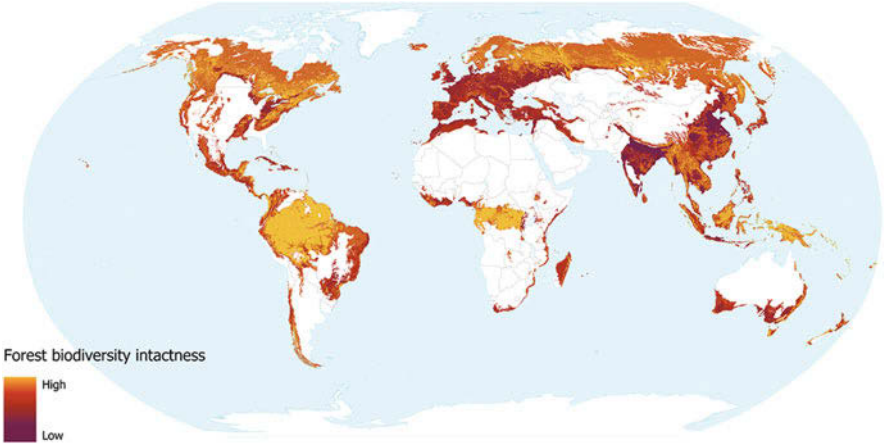

Fig. 6 Forest biodiversity intactness, showing the impacts of forest change and human population density. Yellow shows more intact areas and dark red more degraded areas. After Hill et al. (2019)

and of Canada and Alaska, and in the tropical lowland forest of the northwestern Amazon basin in connection to the Guyana shield.

7 Extent of Forest Degradation

Primary forest losses arise not only from forest destruction in the course of conversion to other land use systems, but also from the continuing degradation of the remaining forests through human activity. The diversity of forest-dwelling species decreases with the extraction of timbers and wood fuel, with forest fragmentation and the occurrence of surface fires, through the decimation of large vertebrates (defaunation), and it may shrink after the invasion of non-native species (Pauchard and Shea 2006). Many of the processes that cause forest degradation are interrelated, and secondary effects are often more destructive than the degradation itself (Zimmerman and Kormos 2012). For example, the frequency of fires increases with the intensity of timber use and the degree of forest fragmentation.

The extent to which forests have been degraded can usually only be approximated, even with modern LIDAR and radar techniques. For the tropical moist forest, it was assumed in 2000 that degraded forests and secondary forests accounted for up to 8.5 million km^2 (more than 75%) of the 11 million km^2 of forest area remaining in that time (ITTO 2002).

The FRA counts 28% of the global forest area as intact primary forest (FRA 2020), and Bryant et al. (1997) 22% as frontier forests; the remaining forest area must be considered as more or less degraded. Woodcock et al. (2015) estimate that globally roughly half of the remaining forests are primarily used for timber production, i.e. around 20 million km^2. According to Mercer (2015), 24% of the remaining tropical forests must be considered intact, 46% as fragmented and the remaining 30% as degraded through human use (e.g., timber extraction, fire). In the Amazon basin, around a quarter of the forest area is used for logging (Bongers et al. 2015). In Africa, around 30–40% of the remaining tropical forests are granted as concessions for timber extraction (Laporte et al. 2007).

Fires are important drivers of forest degradation, especially in the boreal forest, but also in tropical dry and moist forests. In the Brazilian Amazon (4.1 million km^2), 27.5% of the forest is <10 km from areas where it has burned (Peres et al. 2006); the frequency of fire has significantly increased in recent time (Aragao and Shimabukuro 2010).

In the temperate forest biome, the majority of the remaining forest area, in central and western Europe more than 95%, is allotted to managed forests, predominantly even-aged forests with more or less regular thinning operations, which have lost part of their biodiversity. This applies in a similar way to large parts of the subtropical forest biome.

During the last decades, large tracts of the boreal forest have been degraded and fragmented by logging activities and mining and infrastructure projects. More than 50% are subject to more or less intense human interference (Potapov et al. 2008). In Canada's boreal forest, almost a third of the area is destined for forestry (Carlson et al. 2015); 730,000 km^2 are affected by timber use and other human activities (Badiou et al. 2013). Mineral resources are mined in additional forest areas or infrastructure has been established (Carlson et al. 2015). Around 10,000 km^2 of Canada's total forest area of 4.2 million km^2 have been cleared annually in 2010–2020, 21,800 km^2 in 2019 alone (Schindler and Lee 2010; Global Forest Watch 2021). In the southern boreal zone of Canada, only 36% of the forest is intact (Lee et al. 2006).

In the Eurasian taiga, Achard et al. (2006) documented a rapid change in forest cover on 400,000 km^2 due to clearing and on 700,000 km^2 due to increased fire frequency in the 10–20 years before 2005. From 2001 to 2019, about 640.000 km^2 of forest were cut in Russia; part of it is left for natural regeneration (Global Forest Watch 2021). In European Russia alone, 22,100 km^2 (1.5%) of forest were lost in the period 2000–2005 (Potapov et al. 2011). In the boreal forests of Scandinavia, disturbance intensity has markedly increased since 2016, partly due to the recent increase in harvesting intensity (Ceccherini et al. 2020).

8 Drivers of Forest Degradation and Deforestation

Forest degradation can lead to the partial or complete loss of biota with close affinity to the structural elements that are characteristic for old-growth forests, without the loss being reflected in the forest area statistics. The factors driving forest degradation and deforestation vary widely among the forest biomes and forest regions. By far the most important driver in the tropical forest is agricultural expansion, both traditional small-scale shifting cultivation and subsistence farming, and modern marketoriented agriculture (Hosonuma et al. 2012; FAO and UNEP 2020). While oil palm and tree plantations as well as commercial logging are the most important drivers in Southeast Asia's tropical forests (Seymour and Harris 2019; Qaim et al. 2020), this is small-scale farming and firewood extraction in Africa. Commercial logging plays an additional role here. In South America, the expansion of cattle pastures and the cultivation of soy for meat production for urban and international markets are key drivers (deFries et al. 2010; Laurance et al. 2014a, b; Martin 2015; Sloan and Sayer 2015; Curtis et al. 2018; Seymour and Harris 2019).

In the temperate forest biome, agricultural expansion has been the main driver of forest transformation in the past. Historical forest management forms such as coppicing have locally increased forest biodiversity by favoring light-demanding and disturbance-tolerant taxa. In recent time, the most important disruptive factors are widespread forest management for timber production together with atmospheric nitrogen deposition (Nordén et al. 2008; Wallenius et al. 2010; Leuschner and Ellenberg 2017). Modern forest management has nearly completely eradicated the senescence phase of forest dynamics with its large deadwood stocks, and habitat diversity has decreased due to the introduction of largely uniform management regimes, often in form of conifer plantations. Nitrogen deposition, often in combination with artificially high game densities, is increasingly impacting forest biota and tree regeneration in the temperate zone. However, eutrophication is of growing importance also in tropical forests (Homeier et al. 2017).

In the boreal forest, forest degradation is mainly caused by expanding logging activities, frequently on large clear cuts, to supply international markets. Anthropogenic and natural fires and large infrastructure and mining projects have a growing influence on the Eurasian and North American boreal forest (Achard et al. 2002; Potapov et al. 2011; Badiou et al. 2013).

Widespread logging activities and infrastructure establishment in the boreal forest have an impact on the occurrence of large mammals such as woodland caribou, grizzly, wolverine, lynx, wolf and others, which withdraw mostly to the intact forest blocks.

9 Impact of Forest Degradation on Biodiversity

The extent to which selective timber extraction and silvicultural management regimes are reducing the forest-dwelling biodiversity depends on the intensity of the interventions and the resulting changes in forest structure (Putz et al. 2012). Interventions of very low intensity, by which only a few logs per hectare are removed, can even promote the diversity of early-successional taxa, the occurrence of which in old-growth forests is restricted to canopy gaps. In tropical moist forests, the diversity of mammals only started to decrease with a wood removal of 10 m^3 ha^{-1}, that of the amphibians at a threshold of 23 m^3 ha^{-1}, and that of the insects of 41 m^3 ha^{-1}. With a harvest of 38 m^3 ha^{-1} (mammals) and 63 m^3 ha^{-1} (amphibians), the diversity dropped to half of the richness of the primary forest

(Burivalova et al. 2014). After two rounds of selective timber extraction, disturbed forests in Borneo still held 75% of the original diversity of birds and dung beetles at a local scale (Edwards et al. 2011). Carefully executed low-impact logging can therefore leave tropical forest stands with a relatively high diversity, which can play important roles for biodiversity conservation (Bicknell et al. 2014). This is valid especially, when sufficiently large primary forest remnants with intact flora and fauna are left in close neighborhood, and forest access is difficult. However, it must be taken into account that extinction events often only occur with a delay (extinction debt), and that these losses can lead to the progressive impoverishment of exploited forests, particularly in terms of primary forest species with narrow niches (Wilcove et al. 2013). Depending on the organism groups, 5–57% of the forest species in Amazonia occur exclusively in primary forests (Barlow et al. 2007). In addition, exploited forests have a significantly higher fire risk, and forest access tracks increase the hunting pressure with the threat of defaunation (Clark et al. 2009).

Fires occur more often in exploited and fragmented forests because more deadwood is available, the stand microclimate is drier and people setting fires may enter (Aragao and Shimabukuro 2010). In tropical, subtropical, and boreal forests in particular, fire is likely the main driver of forest degradation, causing fundamental change in the communities. Even light surface fires can kill more than 40% of the thin-barked trees in tropical moist forests and trigger a succession toward secondary forests with higher light penetration, in which shorter-lived, fast-growing pioneer tree species dominate, and the primary forest fauna is replaced by stress-tolerant species of secondary forests (Barlow and Peres 2004, 2008). Secondary forests burn more rapidly than primary forests. Repeated fires can eventually lead to the formation of tree savannas, causing the almost complete loss of the characteristic primary forest flora and fauna.

Tropical secondary forests become increasingly diverse with growing age, but the species pool of primary forests is not reached even after several decades (Chazdon et al. 2009). The re-immigration of many primary forest species can take centuries. Transformation systems such as oil palm and rubber plantations, which largely replace the lowland forest in Southeast Asia, have a much lower biodiversity than the primary forest (Barnes et al. 2017). More than 70 or 80%, respectively, of the forest species among birds and butterflies no longer exist in these intensively managed habitats (Koh and Wilcove 2008). A global comparison of the effects of disturbance on ten animal groups and trees showed that, averaged over all organism groups, 41% of the species were dependent on the undisturbed tropical forest (Alroy 2017). Tropical forests are also much richer in symbiontic fungi than tree plantations or other land use systems, in which pathogenic and saprophytic fungi are more common (Brinkmann et al. 2019). This illustrates the irreplaceability of tropical primary forests for biodiversity conservation.

10 Defaunation

Bushmeat hunting has been a serious threat to many tropical forests in recent time (Wilkie et al. 2011; Dirzo et al. 2014; Ripple et al. 2015); larger mammals are particularly affected. This form of degradation is not detected by remote sensing and therefore underestimated. Defaunation due to both chasing and indirect species loss with land use change has multiple implications for forest communities and ecosystem functions (Gardner et al. 2019). One consequence of defaunation is poor dispersal of large-seeded, endozoochorous species and the accumulation of seedlings under the mother tree. In many cases, herbivory and predation patterns change, certain tree species become more abundant, and species diversity generally decreases (Kurten 2013).

Eighty to ninety-six percent of tropical tree species produce fleshy, vertebrate-dispersed fruits, and many Amazonian tree species rely on large frugivore vertebrates as dispersers. Simulation models show that a decrease in this group of animals leads to a reduction in above-ground biomass in tropical forests, since many of the affected, animal-dispersed, large-seeded tree species have high wood densities and achieve large maximum tree heights (Peres et al. 2016). Accordingly, a recent study predicts a decrease in carbon storage capacity of the Brazilian Atlantic rainforest, if defaunation leads to a decrease in vertebrate-distributed tree species with high C stocks (Bello et al. 2015). In Nigeria's lowland forest, the decimation of large primates by hunting has led to changes in the species composition of tree regeneration (Effiom et al. 2013). Changes in regeneration and an increase in tree species with low wood density and in lianas have also been found with intensive hunting of mammals and birds in forests in Panama (Kurten et al. 2015) and Borneo (Harrison et al. 2013). This factor has a major impact on the biodiversity of forests, because the areas affected by defaunation are much larger than the areas only impacted by deforestation or timber extraction (Benítez-López et al. 2019). Remote and protected forest areas are often affected, too.

11 Forest Fragmentation

Habitat fragmentation also contributes to the erosion of forest biodiversity and it may threaten the functionality of forest ecosystems. According to a global meta-analysis, Haddad et al. (2015) reported decreases in forest biodiversity by 13–75% due to fragmentation. The ongoing opening-up of previously untouched forest areas through road construction (Ibisch et al. 2016; Laurance et al. 2017) and the building of access tracks for oil and gas exploration (Finer et al. 2008; Laurance and Arrea 2017) and for the construction of dams (Benchimol and Peres 2015) is a main driver of forest fragmentation. Road construction has resulted in 70% of the remaining global forest area being <1 km distant to the nearest road (Haddad et al. 2015) and almost 20% of the forest area being <100 m from the forest edge (Brinck et al. 2017).

In the three large tropical forest realms, the forest has now been split up into 50–130 million forest fragments (Brinck et al. 2017; Taubert et al. 2018), with the number of fragments increasing with decreasing size (Tapia-Armijos et al. 2015). More than half of the remaining forest in Brazil's Atlantic rainforest is <100 m away from the forest edge (Ribeiro et al. 2009). Many tropical forest reserves that were embedded in a forest matrix at the time of their establishment have now become isolated forest fragments (DeFries et al. 2005; Martínez-Ramos et al. 2016), in which signs of degeneration are increasing.

Various long-term studies have shown that species losses are highest in small, highly isolated fragments and that loss rates depend on fragment size and the mobility of the organisms (Haddad et al. 2015; Gibson et al. 2013: small mammals, Didham et al. 1998: beetles, Feraz et al. 2003: birds). Hansen et al. (2020) showed for tropical forests that the likelihood of further size decrease is highest in the smaller forest fragments. A consequence of fragmentation can be fundamental changes in plant species composition and shifts between different plant functional groups, notably the increase in disturbance-tolerant pioneer tree species at the expense of slower-growing, animal-dispersed late-successional tree species, and increases in lianas (Laurance et al. 2014a, b; Benchimol and Peres 2015; Magnago et al. 2017).

The fringes of tropical forests, estimated at a total length of ca. 50 million km worldwide (Brinck et al. 2017), are exposed to pronounced edge effects, which negatively affect species composition and ecosystem functions up to several hundred meters into the forest (Haddad et al. 2015; Laurance et al. 2002; Pfeiffer et al. 2017). This has been studied, for example, for neotropical rainforest amphibians and reptiles (Schneider-Maunoury et al. 2016). Of 1,673 vertebrate species, 85% showed their frequency to be affected by forest edges (Pfeiffer et al. 2017): Typical forest species (forest core species) only reach their highest abundances several hundred meters from the edge of the forest. Heavily fragmented forests with a high proportion of forest edges are also more frequently exposed to fires (Armenteras et al. 2013).

12 Current Trends in Forest Biodiversity and Species Losses

12.1 Tropical and Subtropical Forests

Tropical forests are home to numerous species with small distribution ranges, which makes them particularly vulnerable. Accordingly, 85% of vertebrate species that have recently become extinct are taxa living in the tropics or overwintering there (IUCN 2018), many of them in forests. The estimated number of 130,000 recently extinct insect species refers mainly to tropical species (Régnier et al. 2015). At least a quarter of all Amazonian tree species are already classified as threatened with extinction according to IUCN criteria (ter Steege et al. 2015).

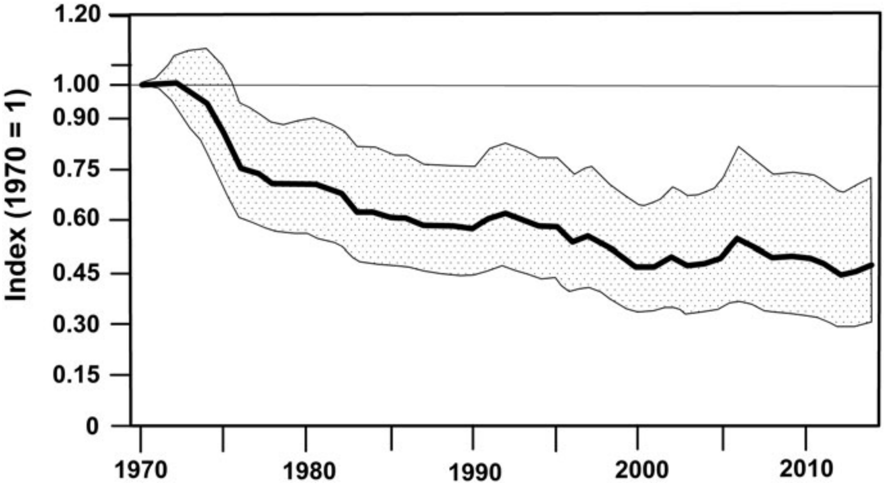

Fig. 7 Change in the Living Planet Forest Specialist Index of the population sizes of 268 vertebrate species from forests (455 populations) between 1970 (= 1) to 2014 from all over the world. Shaded area shows 95% confidence interval of the weighted mean. After WWF (2018)

The best available estimate of global population trends of forest-dwelling organisms is the WWF Living Planet Forest Specialist Index, for which population sizes of up to 268 forest vertebrate species (mammals, birds, reptiles, amphibians) have been continuously recorded since 1970 (WWF 2018). It includes some of the large iconic mammals of tropical forests such as primates and top carnivores. The index has decreased by 53% in the period 1970–2014, i.e. more than half of the vertebrate populations have been lost in <50 years (Fig. 7).

12.2 *Temperate and Boreal Forests*

12.2.1 Site Comparisons

Forest degradation, fragmentation and defaunation result in population declines and regional and global extinction events that have only rarely been documented precisely, because they are difficult to record and often only occur with a delay. The temperate forests of Eurasia and North America, which have long been used for logging and hunting, have lost most of their typical forest fauna of large mammals (e.g., bear, wolf, elk, European bison, aurochs) already in historical times (Vera 2000). Further, multiple studies comparing managed temperate forests to nearby primary forests (or forests with true old-growth characteristics) evidence an impoverished biodiversity with association to dead wood and old and senescent trees in most managed stands. Organism groups with observed reductions in species richness and abundance upon increasing management intensity are saproxylic insects (Müller and Bütler 2010; Jacobsen et al. 2020), saprophytic fungi (Heilmann-Clausen and Christensen 2003; Dvorák et al. 2017), ectomycorrhizal fungi (Tomao et al. 2020), and epiphytic lichens (Nascimbene et al. 2013; Kaufmann et al. 2017) and bryophytes (Friedel et al. 2006; Fritz et al. 2008).

For example, ancient beech forests in the Slovakian Carpathians are home to a 50% greater diversity of epiphytic bryophytes and twice as many epiphytic lichen species than neighboring managed beech forests (Kaufmann et al. 2017). A significant proportion of the about 1,500 species of deadwood fungi in Central European forests are lacking in managed forests with little deadwood (Dörfelt 2007; Härdtle and von Oheimb 2013). In contrast, vascular plant diversity is a poor indicator of old-growthness (Lelli et al. 2019), as diversity is generally promoted by disturbance, at least at low to moderate intensities.

The overall value of primary or old-growth forests for conserving the diversity of these forest-dwelling organisms is broadly accepted. Forest reserves with a management history in the recent past cannot fulfill this task (Meyer et al. 2021), as the biodiversity of managed forests recovers only slowly (Burrascano et al. 2018).

12.2.2 Biodiversity Trends

Biomass and species richness of forest-living arthropods have declined by 41% and 36%, respectively, between 2008 and 2017 in 30 forest sites in Germany (Seibold et al. 2019), paralleling the recently documented insect biomass decline in Central Europe's agricultural landscape (Schuch et al. 2012; Hallmann et al. 2017). Even though the sampling interval covers only 10 years, the results point at landscape-scale drivers of arthropod decline that also impact forests.

More detailed information is available on long-term trends in forest bird populations in Europe's temperate forests. Population monitoring of 34 forest bird species in the forests of England showed an average decrease by 28% in the period 1970–2018; specialist species (22 taxa) declined by even 41%, while generalist species (12 taxa) increased by 7% (Dept Environ. Food and Rural Affairs UK 2020; see Fig. 8). Of 53 bird species in Germany with habitat preferences for forests, 21 show long-term population increases, 12 decreases, and 20 no clear long-term population trend (Gerlach et al. 2019). As in England, increases were observed in generalist species with wide ecological niches and occurrences in non-tree habitats as well. These species seem to profit from local shifts to a more nature-oriented silviculture with more hardwoods, and from eutrophication. Long-term population declines and local extinctions have been observed in bird species adapted to old-growth features such as old trees, high small-scale structural heterogeneity, large deadwood amounts, and long forest continuity. Examples are the white-backed woodpecker, *Dendrocopos leucotos*, gray-headed woodpecker, *Picus canus*, Eurasian woodcock, *Scolopax rusticola*, and capercaillie, *Tetrao urogallus*. Similar observations were made in Switzerland's forests (Grendelmeier et al. 2020).

A resampling study of epiphytic lichens in northwest German broadleaf forests indicated that 28–30% of the species have been lost in the past 100–150 years, likely

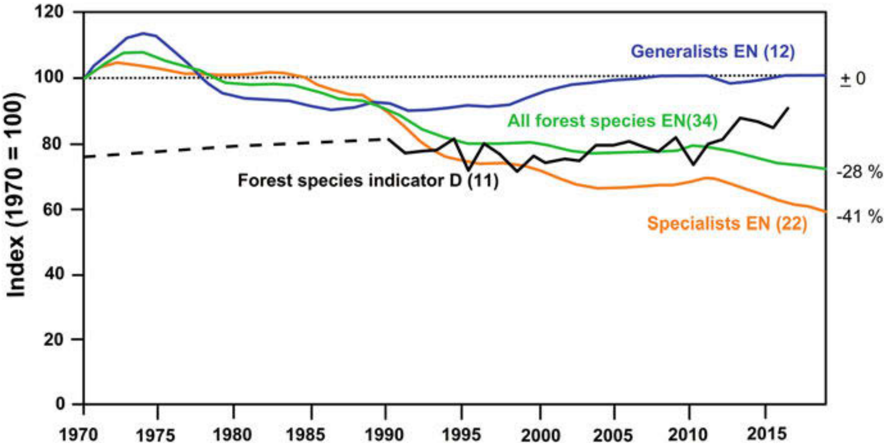

Fig. 8 Results of forest bird population monitoring in England (EN) and Germany (D) since 1970 (index values). The numbers in brackets denote the numbers of studied species. In England, generalist and specialist bird species were monitored separately. After Dept Environment, Food, and Rural Affairs UK (2020), and Bundesamt für Naturschutz 2018 (NBS-Indikator Artenvielfalt und Landschaftsqualität, Teilindikator Wälder, data of DDA 2017)

due to the reduction of deadwood amounts and senescent trees, forest drainage, and nitrogen deposition (Hauck et al. 2013). The fact that the diversity of epiphytic bryophytes is significantly lower in Central European managed forests than in unmanaged forests also points at species losses due to management intensification (Hofmeister et al. 2015). Part of the temperate tree flora is also endangered. More than half of Europe's endemic tree species are threatened (Rivers et al. 2019), notably taxa of the species-rich genus *Sorbus*.

Formerly managed forests taken out of use for conservation purposes achieve the diversity of characteristic forest biota only after many decades, if not centuries. According to a meta-analysis, the epiphytic lichen flora of temperate managed forests takes on average 180 years, the community of ECM fungi 90 years, and the saproxylic beetle fauna 60 years to recover to the state of old-growth forests (Spake et al. 2015). A modeling study based on estimated extinction rates thus assumed that the fragmented production forest landscape of Western Europe (The Netherlands) has lost, or will lose, 13–24% of its native forest-dwelling species, and about half of the original saproxylic species due to low deadwood amounts (Wallenius et al. 2010).

Decade-long nitrogen deposition, together with the abandonment of more traditional management regimes, is a key driver of recent compositional change in the herb layer composition of temperate forests (Verheyen et al. 2012; Förster et al. 2017; Heinrichs and Schmidt 2017). In general, N-demanding, disturbance-tolerant species with wider ecological niches and larger distribution ranges have increased at the expense of small-ranged, more nitrogen-efficient plants with narrower niches (Staude et al. 2020), resulting in pronounced species turnover within a few decades.

In addition, eutrophication together with the shift to a largely uniform forest management has resulted in many temperate forest regions in widespread homogenization of the herb layer vegetation during the last 40–70 years (Heinrichs and Schmidt 2017; Prach and Kopecky 2018). The related plant species richness has remained unchanged (Berrnhardt-Römermann et al. 2015; Staude et al. 2020) or decreased in the last decades (Prach and Kopecky 2018; see also Newbold et al. 2015). In many temperate forests, artificially high game densities have a negative influence on herb layer species richness at the plot and landscape level (Jenkins et al. 2014; Berrnhardt-Römermann et al. 2015).

13 The Biodiversity of Europe's Temperate Forests with a Focus on Germany

Central Europe with more than 7,000 years of permanent human settlements may serve here as an example for assessing the long-term impact of man on forest biota. In Europe, as in eastern North America, only tiny remnants of primary forest remain. The FRA assessment gives for Europe only 41.800 km^2 of primary forest in 2020 (including boreal and Mediterranean forests, but excluding Russia; FRA 2020). Applying stricter criteria, Sabatini et al. (2018) report 14.000 km^2 for Europe (excluding Russia), which is 0.7% of the forest area. These small leftovers are not equally distributed to Europe's different forest types (Sabatini et al. 2020).

In Central and Western Europe, true primary forests are virtually absent. The vast majority of the existing Central and Western Europe forests are today subjected to silviculture, with forest managers selecting tree species, conducting thinning to increase yield, and finally cutting the trees well before the age of senescence. Forests that were set aside in recent time for protecting biodiversity usually bear the legacy of the former production forest for many decades if not centuries, and their biodiversity lacks part of the characteristic old-growth species (Meyer et al. 2021).

The fundamental change in forest biodiversity that took place since humans began to utilize the forest shall be illustrated by the example of Germany's forests. Eight thousand years ago, about 97% of Germany is thought to have been covered by forest. During the Middle Ages, forest cover shrunk to about 20% (Bork 2001), but this figure has increased through re-afforestation to 31% in recent time. Around 23% of Germany's recent forest area represents re-afforestation on arable land and heathland (Glaser and Hauke 2004). In many regions, especially in the lowlands, only small remnants of the so-called historically-old forests have survived, i.e. forests with more than 200 years of forest continuity. Before large-scale forest destruction, about 67% of the forest area was potentially covered by forests of European beech (*Fagus sylvatica*; the rest mainly by oak, pine, and mixed mountain forests; Knapp 2007). The portion of this main native tree species has been reduced to 15.4% of today's forest area. The 16,800 km^2 of beech forest in Germany that still exist today correspond to just 7% of the former potential beech forest area. The natural area share of coniferous forest of <1% (Suck et al. 2014) has been increased by forestry to 54.2%, including above all Norway spruce (*Picea abies* Karst., 25.4%) and Scots pine (*Pinus sylvestris* L., 22.3%) (BMEL 2012). Not only the tree species composition is far from natural, but also the age and population structure of the stands. Most of these are even-aged forests that result from planting or supported natural regeneration and that lack the vertical and horizontal heterogeneity in the canopy that is characteristic of primary forests. 73.7% of the stands are younger than 100 years, only 3.2% older than 160 years. The bulk of Germany's forests thus lack the senescence phase of forest development with dying and dead trees and large amounts of dead wood (Knapp 2007). The average deadwood stock in managed forests is 20.6 m^3 ha^{-1} (BMEL 2012), while in Slovakian primary beech forests, a 10 times higher amount was found (up to 200 m^3 ha^{-1}) (Glatthorn et al. 2017).

Although more than half of Germany was once covered with acidophytic beech forests with low tree species diversity, these forests can be species-rich habitats with a large number of habitat specialists, when management intensity is low. About 15,000 animal species, more than 3,000 macrofungal species and more than 600 lichen species are found in Swiss forests (Bollmann et al. 2009; Scheidegger et al. 2010). Thorough inventories in 60 ha of a beech forest in Hesse (Germany) show the presence of 5–6,000 animal species (Dorow et al. 2010), including numerous specialized saproxylic taxa. Such high species numbers have only been recorded from unmanaged forests.

In 2019, 2.8% of the German forest area had been set aside for the primary goal of biodiversity conservation, i.e. they were given "process protection" and management has ceased (Engel 2019). The area includes national parks, the federal natural heritage, forest protected under the nature conservation law (as far as silviculture is not permitted), and natural forest reserves, the latter often of very small size (at least 0.3 ha). In the early 2000s, beech forests without management covered in Germany 1,226 km^2, which is 2.7% of the current beech forest area or 0.18% of the potential beech forest area in Germany (Knapp 2007). Compared to the global mean of legally protected forest area (11%) and the European average (12%, excluding Russia; FRA 2020), Germany is one of the tail-lights in forest conservation.

14 Effects of Recent Climate Change on Forest Biodiversity

The five primary threats to biodiversity in the world's forests (forest conversion, forest degradation through (over-)use, fires, defaunation, and fragmentation) are exacerbated by recent and to be expected future changes in climate (Hauck et al. 2019; Pörtner et al. 2021). In most forests (apart from the cold-limited northern boreal forests and some mountain forests), a significant temperature increase combined with rising atmospheric evaporative demand exposes trees to stress. This is exacerbated regionally by long-term decreases in growing season precipitation. Atmospheric nitrogen deposition is another important driver of recent changes in forest biodiversity, currently acting mainly in the industrialized regions of the global north, but increasingly expanding to the south as well. In the following chapter, a brief overview of observed major climate change-driven alterations in forest structure and functioning in the forests on earth is given for the period 1980–2020 (for more details, see recent reviews in, e.g., Allen et al. 2010, 2015; Hauck et al. 2019; Forzieri et al. 2021; Pörtner et al. 2021). Many of the anticipated changes have the potential to destabilize forests and to negatively affect forest biodiversity, but the consequences are often not well understood.

14.1 Tropical and Subtropical Forests

In the tropical moist forests, elevated temperatures and more frequent dry spells will very likely cause reductions in tree biomass and long-term carbon sequestration in the future (Mitchard 2018; Maia et al. 2020; Sullivan et al. 2020), caused by increased tree mortality, higher respiration rates, and altered tree species composition (Clark et al. 2010; Anderegg et al. 2015; Doughty et al. 2015; McDowell et al. 2018). Accordingly, carbon storage in the above-ground biomass of tropical lowland forests seems to have increased until 2000 (Baker et al. 2004; Lewis et al. 2009; Qie et al. 2017). Yet, recent studies are indicating a decrease, for example in Amazonia in the period 1983–2011 (Brienen et al. 2015). A shift from longer-lived, slower-growing, drought-sensitive tree species to faster-growing, shorter-lived and more drought-tolerant species is expected (Butt et al. 2014; Esquivel-Muelbert et al. 2018). Some authors have registered an increase in the frequency of lianas in undisturbed tropical lowland forests (Phillips et al. 2002; Schnitzer and Bongers 2011). Possible drivers are the increased atmospheric CO_2 concentration, a higher drought frequency and increased anthropogenic disturbance levels. A higher liana frequency can change the biomass dynamics of tropical forests by influencing tree growth (van der Heijden et al. 2015). Repeated sampling of the avifauna of Amazonian tropical lowland forests in 1980–1984 and again in 2008–2016 revealed reduced abundances of terrestrial and near-ground insectivorous birds even in the absence of deforestation, edge effects, and anthropogenic disturbance, which possibly are caused by climatic change (Stouffer et al. 2021). In the mountain forests of the tropical Andes, a thermophilization of the vegetation has been observed in many places, i.e. an increasing abundance of thermophilic tree species at higher altitudes (Fadrique et al. 2018).

14.2 Temperate and Boreal Forests

In the temperate forest biome, increasing temperatures and rising evaporative demand are seen as the main drivers of widespread vitality loss and increasing mortality of drought-sensitive tree species, especially after extreme hot droughts (van Mantgem et al. 2009; Allen et al. 2010; Anderegg et al. 2013; Leuschner 2020;

Schuldt et al. 2020). Elevated tree mortality rates have been observed in various forest types on the southern and continental edges of the distribution ranges (e.g., in European beech, Penuelas et al. 2007; Lakatos and Molnár 2009), but recently after the extreme 2018/19 drought also in Central Europe (Schuldt et al. 2020; Braun et al. 2021). Widespread tree dieback has also occurred in other temperate regions, e.g., in the *Nothofagus pumilio* forests of Patagonia (Rodriguez-Caton et al. 2016). Long-term growth decline and increased climate sensitivity of growth have been observed since the 1980s in sensitive temperate tree species as European beech even in the center of the species' distribution range (Zimmermann et al. 2015; Knutzen et al. 2017), which is explained by the warming trend and the rising evaporative demand. In some natural temperate mixed forests, a change in the species composition toward more drought-tolerant tree species is taking place (Olano and Palmer 2003; Rigling et al. 2013; Martinez-Vilalta and Lloret 2014). In many North American and European temperate forests, thermophilic herb layer plants have significantly increased in recent decades (thermophilization), while cold-adapted taxa have on average declined (de Frenne et al. 2013).

In the boreal forest, warming has increased growth mainly in the northern taiga, while widespread growth declines and, regionally, increased mortality has been observed in the southern boreal forest and where water is limiting (Dulamsuren et al. 2013; Bond-Lamberty et al. 2014). On the southern edge of the North American boreal forest, *Populus tremuloides* forests have died on large areas as a result of the warming since the 1990s (Peng et al. 2011). An increase in forest fire risk is observed in many regions or is expected for the near future (Malevsky-Malevich et al. 2008). Currently, boreal forests mostly act as carbon sinks, albeit of low strength (Dolman et al. 2012). Yet, this could change with climate warming, especially in drier regions.

In the decades to come, the warming trend may lead in many forest regions on earth to profound changes in community composition, and widespread loss of forest biota with narrow niches is to be expected.

15 A Global Effort to Protect the Last Primary Forests Is Needed

Over the past 8,000 years, humans have reduced the global forest area by almost 40%; a further 40% or more have been degraded through the harvest of wood products, defaunation and fire in such a way that the characteristic forest-dwelling communities only exist in impoverished form (Watson et al. 2018). Fragmentation has resulted in 70% of the remaining forest area being <1 km distant to roads, substantially altering community structure. Today, <11.5 million km^2 of intact primary forest are left, especially in the boreal and tropical forest biome; that is ca. 18.5% of the originally existing global forest area. The remaining forest area free of significant anthropogenic degradation is rapidly declining: Around 30,000 km^2 of

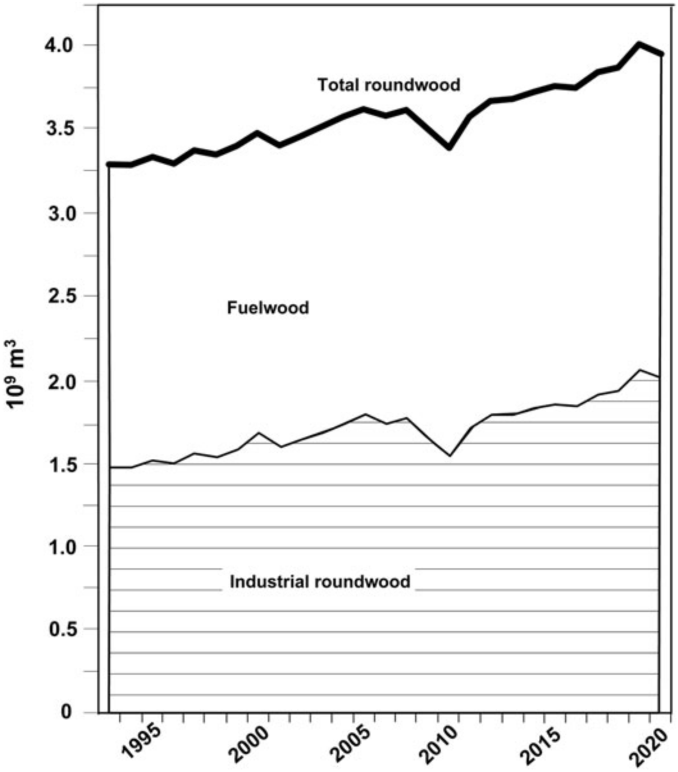

Fig. 9 Total industrial roundwood and fuelwood production on earth 1993–2020. After Forest Product Statistics, FAO. Note that the fuelwood figures likely underestimate the wood extraction for primary energy due to incomplete accounting

primary forest are lost annually through deforestation, and this rate has increased in the last years (Fig. 4). The consequences of rapid warming during the past 40 years (and locally decreasing summer precipitation) are visible in all forest biomes in the form of regionally increasing tree mortality, the thermophilization of vegetation and, in some places, decreasing carbon storage. Progressive warming will further destabilize forests and their biota with likely consequences for productivity, carbon sequestration potential, and other ecosystem functions.

During the last 20 years, the global demand for roundwood (industrial and fuelwood) has increased by on average 0.85% annually to more than 4 billion m^3 year^{-1} in 2020. Demand is expected to rise to about 6 billion m^3 roundwood by 2050 due to population growth and increasing resource consumption levels (Barua et al. 2014; FAO 2021; Fig. 9). Policy recommendations to increase carbon storage in wood products, to replace energy-intensive materials by wood, and to stimulate the use of forest-based bioenergy in order to substitute fossil fuel as part of the green economy (e.g., EEA 2017; Doyle and Roche 2017) will further increase wood consumption and thus demand for roundwood (Searchinger et al. 2018). This would raise the pressure on primary forests, as a growing wood supply from expanding industrial forest plantations and managed forests is unlikely to meet the growing demand (Barua et al. 2014). Promoting the use of forest products and forest management as a pathway to reduce climate warming, as has been agreed on in the Paris Agreement and is implemented into national and supra-national policies such as the European Union's Renewable Energy Directive, is therefore inevitably conflicting with the declared goal of the global community to halt global biodiversity loss (Pörtner et al. 2021).

Currently, about 11% of the remaining forest area has been assigned primarily to biodiversity protection (4.2 million km^2, FRA 2020), but the effectiveness varies greatly from region to region, and much of the left primary forest is insufficiently protected. The future of global forest biodiversity will depend crucially on whether the 11.5 million km^2 of primary forest (i.e., 28% of the remaining forest area or 7.7% of global land surface area) can be effectively protected in the form of large reserves. When a substantial part of the earth's forest-dependent biodiversity is to be inherited to future generations, it is indispensible to establish at least ten effectively managed forest mega-reserves on earth, notably in the boreal forest of Russia, Canada and Alaska, and in the tropical moist forest of northwest Amazonia (Brazil, Peru, Colombia, Venezuela, the Guyanas), of the Congo Basin (Democratic Republic of Congo), and in the highlands of Borneo and New Guinea (PNG and Indonesia). These reserves should cover the complete elevational range of the respective areas to allow for species migration. Only such mega-reserves are suited to buffer forest biodiversity against the negative effects of degradation, fragmentation, and the looming threat of climate change (Watson et al. 2018).

Regional biodiversity hotspots outside these primary forest areas require protected area networks of at least 20% of the forest area to effectively conserve the characteristic forest biota. For forest areas designated mainly for timber production, multiple uses or without designation (i.e., 69% of the global forest area according to FRA 2020), management regimes have to be adopted that guarantee the permanent retention of small areas of intact forest with old and senescent trees and large deadwood amounts. Such a retention approach would allow achieving a certain degree of forest continuity also in logged forests. These remnants might function in the post-harvest forest generation as nuclei of recolonization for disturbance-sensitive taxa (Lindenmeier et al. 2012).

Successful conservation and management of the remaining forests also requires an effective global forest monitoring network, which merges reliable data on forest extent with up-to-date information on biodiversity, forest dynamics processes, and forest ecosystem functions in their dependence on changing environmental conditions. Such a network should link ground-based monitoring in permanent plots (e.g., Malhi et al. 2021) to remote sensing information on forest extent and forest health (as provided by, e.g., Global Forest Watch) in order to understand and control the human impact on forests.

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An Overview on Dendrochronology and Quantitative Wood Anatomy Studies of Conifers in Southern Siberia (Russia)

Alberto Arzac, Marina V. Fonti, and Eugene A. Vaganov

Contents

Abstract Climate conditions affect wood formation during tree growth with consequences for the functioning and survival of trees and forests. Since tree rings are formed at different temporal windows during the growing season, environmental factors will affect the wood structure and function differently. Quantitative wood anatomy (QWA) is a growing field of dendrochronology that allows obtaining a large number of parameters as number, size, and spatial arrangement of xylem cells and structure, highlighting the adjustments of trees to their environment, as well as the relationship between cell structure and function in a spatio-temporal context. This chapter presents an overview of dendrochronological and QWA research in southern Siberia (Russia), a region characterized by a strong water availability effect on tree growth. From this perspective, southern Siberia shares similarities in the climate response of trees growing under Mediterranean climate conditions, an area widely studied by dendrochronologists. Thus, we compiled studies based on

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different approaches (e.g., tree growth climate response, QWA, modeling) and discussed the potential effects of temperature-induced drought on the dynamics of southern Siberia, in particular, the forest-steppe ecotone, as well as the potential consequences for regional and global water and carbon cycles. Therefore, it is expected that climate change will have an impact on tree growth and forest dynamics in the future, with potential ecological and economic consequences for the region.

Keywords Climate, Drought, Modeling, Tracheids, Tree growth, VS-model, Xylem

1 Introduction

Current trends in climate conditions will likely affect woody plants worldwide through rising temperatures (Zhu et al. 2016), increasing atmospheric CO_2 concentrations (Keenan et al. 2014) and temperature-induced drought (Choat et al. 2012). Moreover, the anthropogenic effect is accelerating climate changes (IPCC 2014). Therefore, the evaluation of the ability of woody plants to cope with rapid environmental change is critical to understand the potential effects of climate change on a global scale (McDowell et al. 2008). While growing, trees undergo a continuous process of adjustments to face changing environmental conditions (Fonti and Jansen 2012), defining wood's structure and functioning. However, the rate of adaptability to accelerated changes will vary between species and climate regions, making the dendrochronological studies focused on the effect of climate on tree growth, a powerful tool to unveil the effects of climate across the globe.

Tree-ring and xylem traits-based chronologies are widely used as climate proxies due to their advantage of encoding environmental information at different temporal scales, from inter-annual to intra-seasonal resolution (Bryukhanova et al. 2013; Vaganov et al. 1999). Tree-rings and xylem traits provide valuable information to identify the main factors controlling tree growth, reconstruct past climatic conditions, or evaluate forest dynamics (Vaganov et al. 2006). The information encoded in tree-ring traits has significant implications on global climate, carbon and water dynamics due to forests' critical ecosystem services. Dendrochronology (i.e., the study of tree rings) as a science has a long history. For example, already in nineteenth century patterns among tree rings were used to reconstruct past climate (Twining 1833). Subfields of dendrochronology as the quantitative wood anatomy (QWA), which aims to explore the variability of xylem anatomical traits of trees, shrubs, and forbs, and unveil the links between structure, function, and environment of different xylem traits in terms of plant growth and functioning (von Arx et al. 2016) has more recent origin and is in constant evolution.

Tree secondary growth (wood formation) is driven by environmental factors occurring before and during the growing season (Fonti and Jansen 2012). In vascular plants, the secondary xylem (root, stem, and branch wood) is a complex tissue involved in the transport (e.g., water, nutrient, and hormone), structural support and reserves storage (Olano et al. 2013). The secondary xylem is produced from the vascular cambium in a succession of steps, including the cell division, cell expansion, cell-wall thickening, programmed cell death, and heartwood formation (Rathgeber et al. 2016), which are controlled by exogenous and endogenous factors during the growing season (Hsiao and Acevedo 1974). During this process, xylem is developed toward the pith and the outer living part of the xylem forms the sapwood, where most of the transport and storage occur (Speer 2010). In conifers, up to 90% of the xylem is constituted by individual tracheids (Hacke et al. 2015; Vaganov et al. 2006).

In boreal regions, tree growth occurs from late spring to late summer leading to changes in the tracheids, from wide and thin-walled earlywood cells to narrow and thick-walled latewood cells, oriented toward different functions. Thus, earlywood cells are specialized in efficient sap transport, whereas latewood cells provide mechanical stability (Björklund et al. 2017; Cuny et al. 2014). Moreover, xylem structure highly depends on the timing and magnitude of the environmental factors occurring during the cell developmental phases (e.g., Castagneri et al. 2017; Björklund et al. 2017), leaving a permanent print in xylem traits on a weekly to seasonal temporal scale (Bryukhanova et al. 2013; Vaganov et al. 1999). When dendrochronological studies focus on evaluating cellular structures (as tracheids), QWA becomes the most powerful tool based on the analysis of wood anatomical thin sections.

The preparation of high-quality wood anatomical thin sections is an arduous work that includes several consecutive steps (Gärtner and Schweingruber 2013; von Arx et al. 2016), and QWA analysis has been limited by the quality of the anatomical preparations as well by the acquisition of high-resolution images in the past. However, QWA has experienced rapid development in recent times thanks to the improvement of the preparation techniques of the samples as embedding media and polishing of blocks and sections (e.g., Arzac et al. 2018a; Rossi et al. 2006; von Arx et al. 2016), the evolution of best quality image acquisition systems, and the development of new image recognition software (e.g., ROXAS; von Arx and Carrer 2014) allowing the study and better understanding of wood cell structures. Nowadays, the potential of QWA extends to the study of many xylem traits, including cell number and cell size (e.g., Vaganov et al. 2006), ray parenchyma features (e.g., Fonti et al. 2015; Olano et al. 2013), the spatial arrangement of cells within the tree ring (e.g., Cuny et al. 2014) and its links with function and environment (e.g., Sviderskaya et al. 2021).

Since wood has a tridimensional structure, it might be examined in three different views or cutting planes (i.e., transversal view, radial view, and tangential view) showing a different perspective of the cellular wood structure. Thus, the transversal view provides a cross-sectional view of the tracheids, whereas the radial view shows the total length of the tracheids and the tangential shows a side view of the wood structure parallel to the pith column (Speer 2010). The transversal view is the most commonly used in QWA since it provides a complete vision of cell lumen and cell walls, allows the clear identification of earlywood and latewood cells (Denne 1989), and works over long temporal series by recognizing tree-ring borders (von Arx et al. 2015).

Climatic factors constraining tree growth in temperate and boreal environments shift along gradients, moving from water shortage at lower altitudes/latitudes to temperature limitation at higher. Therefore, the temperature is a primary limiting factor controlling tree growth in cold environments (Vaganov et al. 2006). There is a large body of literature based on different dendrochronological approaches carried out in temperature limited northern latitudes of the Russian territory. The studies cover tree growth response to climate, quantitative wood anatomy, wood density, isotopes analysis and modeling (e.g., Arzac et al. 2018b; Briffa et al. 1998; Churakova et al. 2020; Tychkov et al. 2019; Vaganov et al. 1999, 2006). Although most Russian dendrochronological research has been focused on the northern temperature-limited regions, southern Siberia has been less studied. In comparison to the northern latitudes, tree growth in southern Siberia is mainly controlled by water availability (e.g., Arzac et al. 2018b; Fonti and Babushkina 2016), similarly to Mediterranean environments where the growing season is constrained by summer drought (e.g., Arzac et al. 2016). Therefore, this chapter aims to introduce the fundamental concepts and methods in dendrochronology and quantitative wood anatomy, providing a framework for the studies carried out in southern Siberia (Russia, from 50° to 56° north, and from 59° to 108° east). Finally, the chapter is structured in three main parts: (1) an exploration of the Russian boreal forests, climate, and vegetation (with particular emphasis in southern Siberia); (2) an overview of the main methods applied in dendrochronology and quantitative wood anatomy; and (3) a compilation of dendrochronological studies carried out in southern Siberia.

2 Russian Boreal Forests and Southern Siberia

The Russian boreal forests are the world's largest continuous forest area, representing about 50% of all boreal forests (Krankina et al. 1997). Due to the extension of Russian boreal forests, they stored an estimated 46–67 petagrams of carbon in living biomass (Houghton et al. 2007; Shvidenko and Schepaschenko 2014), highlighting the importance of this ecosystem on a global scale. The climate conditions in the Russian boreal forest are predominantly subarctic (Dfc and Dfd) according to Köppen–Geiger climate classification. However, instrumental meteorological data show significant warming from the eastern Urals to the Pacific coast during the last five decades (Fig. 1a), whereas precipitation changes have been more heterogeneous during the same period (Fig. 1b).

In terms of vegetation, there is a clear latitudinal distribution of different ecosystems within the Russian territory. Thus, from north to south might be classified into four major biomes: (1) the treeless tundra with the presence of permafrost; (2) the taiga dominated by conifer forests (e.g., pine, larch, fir, spruce); (3) mixed forests (e.g., spruce, pine, birch); and (4) the meadow steppe constituted mostly by grasses.

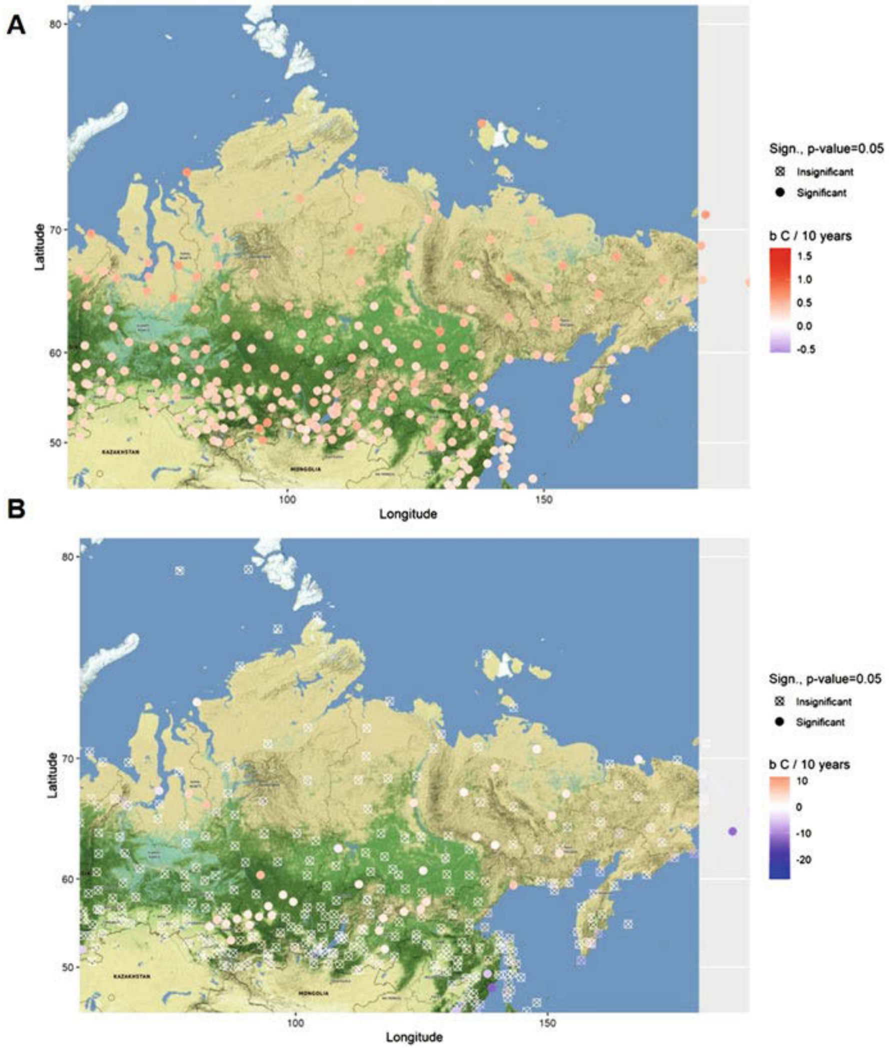

Fig. 1 Climate trends (bC/10 years) in the Russian territory (from eastern Urals to the Pacific coast) for the 1960–2017 period. Climate data were obtained from the meteorological stations available at www.meteo.ru. Mean annual temperature (**a**) and total annual precipitation (**b**) trends. The rate of change is expressed as bC/10 years

In addition to these major biomes, the ecotones in the border between biomes are also relevant. This chapter will mostly focus on one of these ecotones in particular, the transitional area between the humid taiga in the north and the dry steppe in the south (the southern forest-steppe ecotone), characterized by a mosaic of forest patches (e.g., pine, larch, birch) on the meadow steppe (Mueller et al. 2016).

The forest-steppe occupies a vast territory from central Europe in the west to southeastern Siberia in the east (Chibilyov 2003), whereas the southern Siberia forest-steppe ecotone covers about 14% of the Russian territory (Smelansky and Tishkov 2012). In general, the forest-steppe is a limited water environment. Therefore, projections of above-average warming in the region (Groisman et al. 2012) might trigger temperature-induced drought (Dai et al. 2004), compromising forest patches dynamics in terms of phenology, growth rates, physiology, and geographical distribution (Tabakova et al. 2020; Tchebakova et al. 2016), with potential consequences on both regional and global climate dynamics as well as on water and carbon cycles (Gustafson et al. 2011; Shvidenko et al. 2012).

3 Methods in Dendrochronology and Quantitative Wood Anatomy

The general process working on QWA involves the imaging and analysis techniques in a series of successive steps, each of them being decisive for the next: (1) samples collection (common to tree-ring width analysis); (2) preparation of permanent wood thin sections of 10–15 μm thickness; (3) thin sections digitalization; and (4) measurements and analysis (Gärtner and Schweingruber 2013; von Arx et al. 2016). All these stages, in turn, are divided into others of diverse complexity.

3.1 Sample Collection

QWA might be applied to different plant organs (e.g., stem, branches, roots, etc.), and the methods of sample collection will vary according to the aims of the study or the tissue under analysis (von Arx et al. 2016), similarly to tree-ring research (Kirdyanov et al. 2018). In xylogenesis studies, micro-cores are sampled weekly during the growing season by using a Trephor (Rossi et al. 2006), or after the growing season, beyond the wounding of the cambium, with a needle followed by localization of the wound-associated tissue modification (pinning; Wolter 1968). In tree-ring and QWA studies focused on long temporal series (several years), wood samples are taken with an increment borer, varying in length (from 20 to 60 cm) and diameter (5, 10, 12 mm). Stem wood cores are taken in a radial direction, from bark to pith, and perpendicular to the tree stem (i.e., perpendicular to the axial direction of the tracheids in conifers). However, wood samples could also be obtained from stem, branches, and roots discs, from which a radial section is extracted. During the sampling, it is critical to collect the material properly since mistakes occurring at this stage can compromise the final results.

3.2 *Preparation of Thin Sections*

The preparation of wood histological thin sections has evolved in recent years, with significant changes in the protocols followed. Thus, the most common method consists of the sectioning of 15 μm thick sections using a sledge microtome (Gärtner and Schweingruber 2013). When sectioning with a sledge microtome, corn/rice starch could be added to the wood's surface to increase the cell structure stabilization and to avoid potential damages caused by the microtome blade. However, more sophisticated techniques can also be applied, for example, the infiltration and embedding of wood material in paraffin to be then sectioned in a rotary microtome, following the protocol used for xylogenesis studies (Rossi et al. 2006). The infiltration and embedding in harder resins could also be applied (Arzac et al. 2018a). Independently of the method, the objective is to achieve high-quality thin sections (von Arx et al. 2016). The resulting sections are stained with a solution of Alcian blue (or astra blue) and safranin to obtain a differential staining (Gärtner and Schweingruber 2013), resulting in lignified cells turning red and non-lignified turning blue. Finally, thin sections should be digitalized using a digital camera mounted on an optical microscope or, more recently, using a slide scanner (von Arx et al. 2016), with objectives of 10X in the case of conifers.

3.3 *Measurement and Analysis*

The complexity of measurements increases with the xylem trait to be analyzed, from tree-ring width to cellular structure. Thus, tree-ring, earlywood and latewood width are relatively easier to measure either by direct observation of the wood samples using specialized tree-ring measurement systems (e.g., LinTab, Velmex) or by measurement of digitalized wood samples (e.g., CooRecorder/CDendro, WinDENDRO). In the case of QWA, the techniques may also vary according to the level of detail, from the measurements of few xylem traits (e.g., cell number, lumen diameter, and cell-wall thickness) along individual cell rows in semiautomatic software such as AxioVision (Carl Zeiss, Jena, Germany), Lineyka (Silkin 2010), ImageJ (Schneider et al. 2012), or in a more automatic way as AutoCellRow (Dyachuk et al. 2020). Cell data from 5 radial rows are often used for the tracheidogram method analysis (Belokopytova et al. 2019; Vaganov 1990; Fig. 2). However, measurements performed along rows of tracheids are more orientated toward cell morphological characteristics than an ecological perspective. On the other hand, automatized tools as ROXAS (von Arx and Carrer 2014), combined with Image-Pro Plus (Media Cybernetics, Rockville, MD, USA), allow the automatic identification of all the tracheids and tree-ring borders contained in a given surface of plant tissue, providing a more extensive range of parameters (e.g., cell number, lumen diameter, cell-wall thickness, theoretical maximum water conductivity, anatomical wood density and estimation of carbon accumulation in cells, among others; Fig. 3).

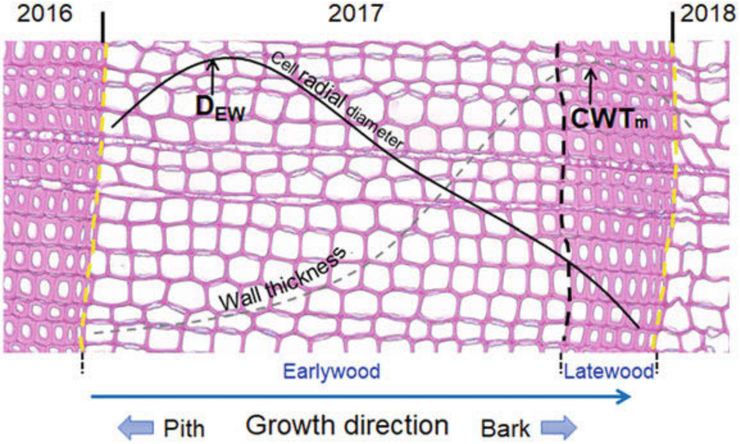

Fig. 2 Schema of a *Pinus sylvestris* xylem transversal view showing the tracheidograms for cell radial diameter (solid line) and cell-wall thickness (dashed line). Yellow and black dashed lines show the tree-ring borders and the earlywood and latewood zones, respectively. D_{EW} maximum cell radial diameter in the early wood, CWT_m maximum cell-wall thickness in the latewood

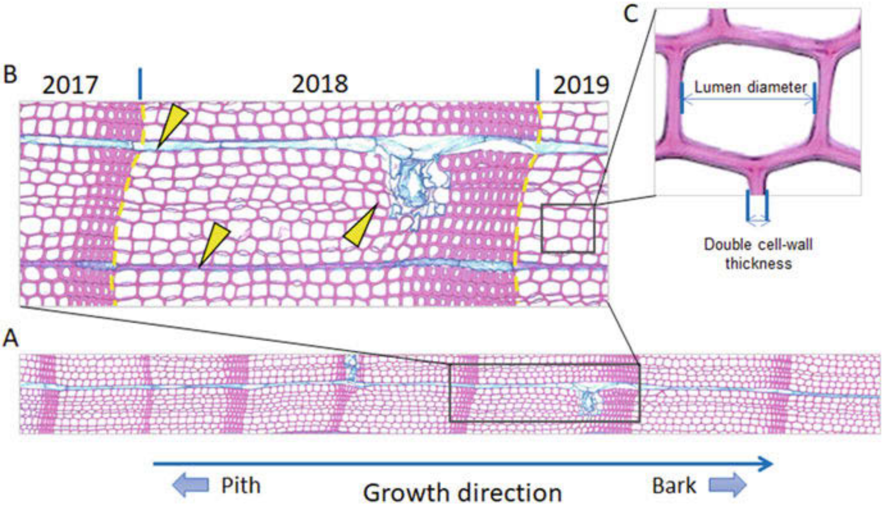

Fig. 3 Overview of a 12 μm thin transversal section of *Pinus sylvestris* including several tree annual rings (**a**); magnification of an individual tree-ring (**b**); and zoomed-in details of tracheids (**c**). Tree-ring borders are defined by the yellow dashed lines, up triangle shows a resin duct and down triangles show ray parenchyma

Once tree-ring width and xylem traits parameters are measured, different statistical techniques can be applied to extract the encoded information in each parameter at different temporal resolutions. Pearson's correlation between xylem traits and climate data (e.g., mean monthly temperature and total monthly precipitation) is the first step in order to obtain monthly resolved information. However, if a higher temporal resolution is required, moving correlation between the xylem traits and daily climate data (e.g., mean daily temperature and total daily precipitation) can be applied in different windows and different day steps (e.g., Babushkina et al. 2019a; Belokopytova et al. 2020). In the case of the output from ROXAS, a detailed analysis can be performed. Thus, for example, a tracheidogram can be produced by the use of the package "RAPTOR" (Peters et al. 2018) for R, or the analysis could be performed dividing the tree ring into different proportioned sectors to unveil the information at different temporal resolutions (Castagneri et al. 2017). More complex statistical methods as generalized additive mixed models (GAMM; Zuur et al. 2009) allow assessing the effect of different variables simultaneously on a particular xylem trait.

The process-based Vaganov–Shashkin model (Vaganov et al. 2006) can be applied via a friendly user interface, the VS-oscilloscope (Babushkina et al. 2019b; Shishov et al. 2016; Tychkov et al. 2019), to simulate tree growth of coniferous species as a function of climate conditions (day length and daily temperature and precipitation). In addition, the size of xylem cells (in the form of tracheidograms) and the timing of their formation might be obtained as output data of the model (Popkova et al. 2018).

4 Current State of Dendrochronology and QWA in Southern Siberia

Although late spring and early summer temperatures are generally considered the main driver of tree growth, water availability has a critical role in the southern Siberia forest-steppe (Arzac et al. 2018b; Babushkina et al. 2017; Demina et al. 2017; Tabakova et al. 2020). This is relevant considering that the region might become drier in the future due to temperature-induced drought, highlighting the importance of evaluating tree growth responses to climate in the southern Siberian forest-steppe. Here, we compiled relevant studies of conifer trees in the presented ecotone (Table 1). The studies cover climate-growth responses (e.g., Tabakova et al. 2020), xylogenesis (e.g., Antonova and Stasova 1993; Fonti et al. 2020; Kalinina et al. 2019), quantitative wood anatomy (Arzac et al. 2018b; Babushkina et al. 2019a; Belokopytova et al. 2019, 2020; Fonti and Babushkina 2016) and modeling (Arzac et al. 2018b; Babushkina et al. 2019b; Popkova et al. 2018; Tychkov et al. 2019). In general, most of the studies focus on temporal series starting from 1960 due to the climate data (mostly precipitation) is robust enough for the analysis, and most of them are located relatively in the same region (Fig. 4) covering different

Table 1 An overview of studies related to dendrochronology and wood anatomy carried out on conifers growing in the Siberian forest-steppe

Area	Coord.	T (°C)	P (mm)	Alt. (m)	Sp.	Proxy	Found	Source
Krasnoyarsk Krai	56°N, 92°E	1.3	537	164	*PISY*	QWA	Temperature influence on different phases of xylogenesis and cell-wall biomass accumulation, with EW and LW cells forming at different temporal windows	Antonova and Stasova (1993, 2015)
					PISY	RW, EW, LW, IADF	Influence of early-summer precipitation on RW and EW, and spring temperature on LW Low frequency of IADF	Arzac et al. (2021a)
					PIOB *PISI*	RW, EW, LW, QWA	The analysis of growth and anatomical structure of xylem was performed in three variants of soil moistening and under control parameters	Kuzmin et al. (2011)
					PISY *PISI*	QWA	Analysis of the anatomical characteristics of tree-rings of the PISI scions and PISY rootstocks in relation to the reference (ungrafted) trees	Darikova et al. (2013)
	55°N, 92°E	−0.1	660	200–536	*PISY* *LASI*	QWA	The influence of air and soil temperature on the beginning of xylogenesis of both species. Species specificity and site conditions affect QWA	Kalinina et al. (2019)
					PISY	RW, VS-model	Precipitation favored RW in June, whereas growing season starts at the DOY 142	Arzac et al. (2021a)
Republic of Khakassia	54°N, 89°E	1.1	260	450	*PIOB* *PISY* *LASI*	QWA	Cell parameters of trees from different topoecological conditions allow revealing "key" intervals of season, when climatic factors can be evaluated quantitatively	Babushkina et al. (2018)
							Decrease in EW tracheid lumen and cell-wall thickness with increasing temperature and drought	Fonti and Babushkina (2016)
					LASI	QWA, modelling	The developed model uses isometric scaling within conduits elements to facilitate the assessment of wood hydraulic properties	Sviderskaya et al. (2021)

	54°N, 91°E	1.0	330	660	*PISY*	QWA	EW and LW cell characteristics respond to climate conditions at different temporal resolution in dry environments	Belokopytova et al. (2019)
					LASI	QWA, EW, LW	The effect of EW and LW cell production and cell sizes to RW variability	Popkova et al. (2020)
	53°N, 91°E	1.1	328	300	*PISY*	QWA	Tracheid size is sensitive to drought at different temporal windows	Arzac et al. (2018b)
						IADF	Similar RW and number of tracheids for young and old trees, and differences in the cell-wall thickness but not in the cell radial diameter	Fonti et al. (2020)
						QWA	Independent climate signal of ray parenchyma-based chronologies	Tabakova et al. (2021)
							IADF frequency over 10%	Arzac et al. (2021a)
						VS-model	Variability of size and structure of pine tree-rings due to climatic conditions and growth rate	Popkova et al. (2018)
Western Sayan Mountains	52°N, 91°E	NA	NA	520–1,320	*PIOB*	QWA	Altitudinal anatomical patterns revealed interaction of intrinsic and external factors in the regulation of tracheid differentiation	Babushkina et al. (2019a)
					PISY *PIOB*	QWA	Fluctuations in LW cell-wall thickness in wide rings respond to climate conditions before cell-wall deposition	Vaganov et al. (2020)
					PISY	RW, EW, LW	The role of slope orientation and elevation temperature gradient for pine growth	Zhirnova et al. (2019)
	53°N, 91°E 52°N, 91°E	NA	NA	250–1,350	*PISY*	QWA	Wood formation was interconnected within each zone of the ring, as well as between EW and LW. EW-parameters tend to have more distinctive climatic responses and lower intercorrelations	Belokopytova et al. (2020)

(continued)

Table 1 (continued)

Area	Coord.	T (°C)	P (mm)	Alt. (m)	Sp.	Proxy	Found	Source
Southern Urals and Altay	53°N, 60°E 53°N, 83°E	3.04 2.33	383 430	398 208	*PISY*	RW, EW, LW	Tree-ring traits are sensitive to drought increasing with continentality	Tabakova et al. (2020)
	50°N, 89°E	3.0	152	2,280–2,340	*LASI*	RW, EW, LW, QWA	Warming favors wider EW-cell lumen, thicker LW-walls, denser maximum latewood, and wider RW. Long-term analyses indicated a diverging trend between lumen and cell wall of EW and LW	Fonti et al. (2013)
Baikal	52°N, 104°E 53°N, 105°E 53°N, 107°E 55°N, 109°E	−1.1 −1.7 −1.0 −2.3	377 353 249 352	445 901 452 480	*PISY*	RW, EW, LW	Climate sensitivity decrease with the proximity to a body water mass, being RW less sensitive to climate conditions in coastal sites	Arzac et al. (2021b)

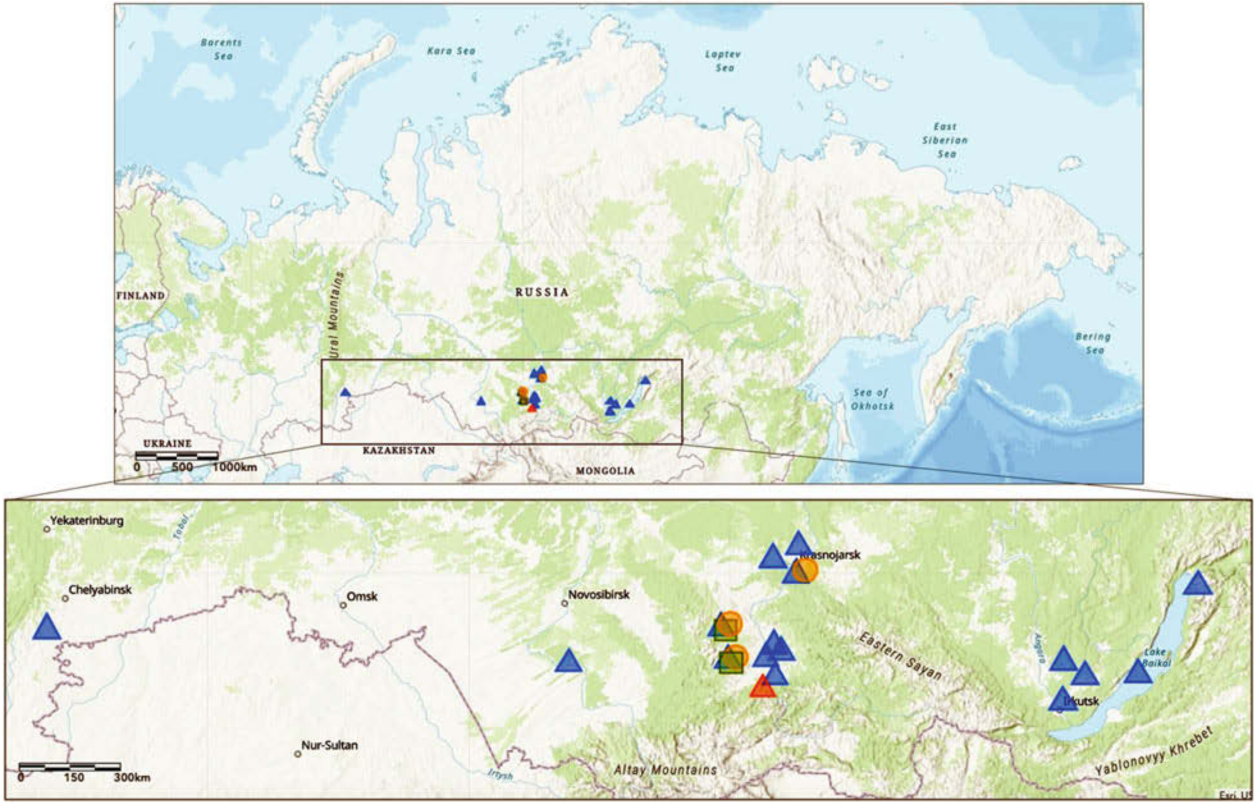

Fig. 4 Distribution of sampling sites (according to the sampled species) related to dendrochronological and quantitative wood anatomy research carried out in southern Siberia. Because some sites have been used in more than one publication, the map shows only once each site to avoid overlapping. Blue triangles, *Pinus sylvestris*; red triangle, *Pinus sibirica;* yellow circles, *Larix sibirica*; green squares, *Picea obovata*

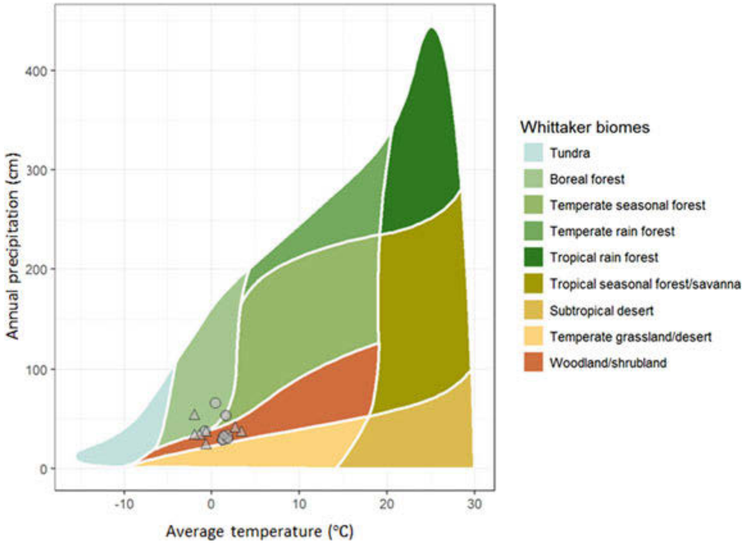

Fig. 5 Distribution of the studied sites in southern Siberia based on Whittaker's biome classification scheme accordingly to mean annual temperature and amount of total annual precipitation. Triangles show climate-growth response studies (tree-ring, earlywood and latewood width); diamonds show QWA studies and circles studies on modeling (VS-model)

biomes (accordingly to the mean annual temperature and total annual precipitation (Whittaker 1975); Fig. 5). Other areas of southern Siberia (e.g., Altay mountains and Buryatia, Belokopytova et al. 2018; Kharuk et al. 2013) have been investigated; however, we focus on areas in which more than one dendrochronological approach has been applied. Despite studies on deciduous species have been published (e.g., Belokopytova et al. 2020), most of the studies are based on conifers (e.g., *Pinus sylvestris* L., *Pinus sibirica* Du Tour. *Picea obovata* Ledeb. *Larix sibirica* Ledeb.) due to the spatial arrangement of tracheid cells, which facilitates the measurements and analyses.

In Russia, studies on stem xylem formation are widespread and have a long history (e.g., Alexandrov 1936; Vaganov et al. 2006). In the forest and forest-steppe zone of southern Siberia, seasonal tree growth of conifers in wet years is characterized by a single-top curve and one continuous period (Antonova and Stasova 1993; Fonti et al. 2020; Kalinina et al. 2019; Vaganov et al. 2006). In years with a pronounced drought within the vegetation period, tree growth slows down and is restored only after a sufficient amount of atmospheric precipitation. In this case, trees form "false rings" (IADF, intra-annual density fluctuation), characterized by a bimodal growth curve (Popkova et al. 2018; Vaganov et al. 2006). A recent study (Arzac et al. 2021a) has shown that IADF frequency in *Pinus sylvestris* is similar to the frequency reach by Mediterranean conifers (Arzac et al. 2018c; Olano et al. 2015).

The growing season in southern Siberia starts at the end of April and cease in September- beginning of October and lasts 118–125 days in southern taiga (Kalinina et al. 2019), 140 days in the forest-steppe zone of southern Siberia (Antonova and

Stasova 2015), and 160 days in pine forests of Minusinsk depression (Fonti et al. 2020). Climate-growth studies show a clear effect of late-spring (May) precipitations triggering tree growth (Arzac et al. 2018b; Fonti and Babushkina 2016) although the timing of climate signals might shift according to the study area (Arzac et al. 2021b; Tabakova et al. 2020). Thus, northern sites tend to have later climate signals than southern sites (Arzac et al. 2021a). Simultaneously, the positive effect of precipitations is coupled with a negative effect of temperature occurring at the same temporal window, suggesting a negative influence of drought. Similarly to precipitation, temperature control on tree growth might shift in time and effect, from positive to negative, with decreasing latitude and altitude (Belokopytova et al. 2020).

Since earlywood and latewood are formed at different timing during the growing season, they respond to climate conditions occurring at different temporal windows (Arzac et al. 2018b; Popkova et al. 2020; Zhirnova et al. 2019). However, as the earlywood constitutes about 70% of the tree ring in some conifers as *Pinus sylvestris* in the forest-steppe (Tabakova et al. 2020), its climate response is closer to the climate response of the total ring. Different species also show different climate sensitivity, being *Pinus sibirica* the less sensitive (Zhirnova et al. 2020). Interestingly, depending on the site, tree species are becoming less sensitive to climate conditions in general, linked to climate trends.

The results of a study of tree-ring anatomical structure of coniferous tree species (*Picea obovata*, *Pinus sylvestris*, and *Larix sibirica*) in the forest-steppe ecotone, where radial growth is limited in the beginning and end of the growing season by air temperature and in the middle of the season by the amount of precipitation (Tychkov et al. 2019), indicated a general decrease in tracheid lumen size in the earlywood zone and cell-wall thickness with increasing air temperature and drought, regardless of species and local growing conditions (Fonti and Babushkina 2016). In particular, a temperature increment of one degree Celsius during the summer months resulted in a 5% decrease in earlywood cell lumen and cell-wall thickness. Consequently, the decrease in hydraulic efficiency was not accompanied by an increase in hydraulic safety, which may have been caused by the insufficient substrate for xylem cell building. Based on these results, we can assume that more frequent and prolonged dry periods may prevent the formation of optimal functional xylem structure in coniferous tree species under these growing conditions.

Multi-year chronologies of tree-ring anatomical parameters of Siberian larch obtained for high altitude areas of Altay Mountains (Fonti et al. 2013) revealed that warmer growth season conditions contribute to the formation of tracheids with larger lumen size in earlywood, thicker cell walls in latewood (and higher wood density), and wider tree rings in general. Differences in inter-annual variations in tracheid lumen size and cell-wall thickness in early- and latewood suggested that changes in xylem parameters in latewood increase mechanical strength and accompany an increase in tree size under more favorable climatic conditions.

5 Conclusions

Tree growth in southern Siberia occurs during the short but hot summers and is likely to be affected due to temperature-induced drought in the future, constraining tree growth by soil moisture availability. Conifers as *Pinus sylvestris* have shown large plasticity in their xylem, allowing them to cope with changing environmental conditions. However, potential changes in climate condition might exceed the adaptability of such species to dry conditions, affecting forest structure, age, and composition and affecting their involvement in critical roles as carbon sequestration. Evidence suggests that some species are more vulnerable than others, exhibiting tree decay and changes in their distribution. Therefore, it is crucial to continue investigating the effects of environmental conditions on tree growth and wood structure to get a deeper understanding of tree responses to changing environment. The combination of multiple discipline as dendrochronology, quantitative wood anatomy, and modeling, with tools such as remote sensing or in situ tree responses (e.g., sap flow, stem diameter fluctuations) will contribute to making better decisions in terms of forest managing to face climate change.

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Holobionts in the Plant Kingdom

Ulrich Lüttge

Contents

Abstract A holobiont is a host organism in interaction with all associated microorganisms as an entity for selection in evolution. Holobionts are akin to symbioses in the broad sense comprising mutualism or parasitism, according to De Bary (Die Erscheinung der Symbiose, 1879). At higher scaling levels, beyond the host-organism level, holobiont-like systems (HLSs) are emergent biological systems in interaction with all their biological components as being subject to evolutionary selection. Botanical examples of holobionts are described ranging from protists to Higher Plants and evaluated as units of evolutionary selection separate from the species concept. HLSs dominated by plants are populations, ecosystems, biomes,

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and the entire biosphere or HLS-Earth. The evolution of HLS poses theoretical challenges beyond neo-Darwinism which are treated in a different article in this volume of Progress in Botany.

1 The Holobiont Phenomenon and Concept

Eukaryotic Higher Organisms live in close association together with microorganisms, comprising bacteria, archaea, fungi, and protists. For instance, we as humans carry 1–2 kg of prokaryotic bacteria with us, with a large diversity of species. They inhabit our skin, mouth, intestine, and other moist surfaces of our body. The molecular size of their genome is up to 2000 fold the protein coding genes of our own eukaryotic genome (Sandoval-Motta et al. 2017a). The ensemble of the microorganisms is named the microbiome. The genetic information of the microbiome together with that of the host is called hologenome (Zilber-Rosenberg and Rosenberg 2008; Rosenberg and Zilber-Rosenberg 2018; Madhusoodanan 2019). The phenomenon has led to the concept of the *holobiont* as *a host organism (plant or animal) in interaction with all associated microorganisms as an entity for selection in evolution* (Matyssek and Lüttge 2013; Vandenkoornhuyse et al. 2015; Rosenberg and Zilber-Rosenberg 2018). The holobiont currently attracts increasing attention in the biological literature.

The microbiome supports host fitness. In humans it is essential for digestion, and it stabilizes the immune system and other functions. In plants the microbiome enables for a wide range of different beneficial effects (Hassani et al. 2018), including life-vigor and ecophysiological performance (Lau and Lennon 2011; Liu et al. 2019). This was also demonstrated experimentally by host-mediated microbiome-engineering (Mueller and Sachs 2015). Different parts of the microbiome can have beneficial or detrimental influences on ecophysiological performance. The large phylogenetic diversity of microorganismic agents in the microbiomes within plants is described by Hassani et al. (2018).

Thus, we may regard holobionts akin to symbioses in the broad sense according to De Bary (1879), overarching mutualism and parasitism. Such a view becomes useful in the comparative biology of holobionts, when we extend the holobiont definition beyond Higher Organisms (animals and plants) as hosts and associated microorganisms toward *a specific organism in interaction with all associated organisms* (Matyssek and Lüttge 2013). Consequently, the extended definition covers all symbiotic associations sensu De Bary as holobionts.

2 Holobionts in the Plant Kingdom

2.1 Cryptogams

2.1.1 Protists: Unicellular Partners in Holobionts

The biodiversity of symbioses is manifold between unicellular partners, which may be green and photosynthesizing (cryptogamic plant-type) and non-green (animal-type), respectively (Karakashian 1975; Smith and Douglas 1987; Reisser 1992). The latter type provides CO_2 and minerals, whereas the former as endosymbionts deliver photosynthetic products. Such systems are holobionts although we can distinguish heterotrophic and autotrophic partners rather than hosts and associated microbes. Examples are ciliates, such as *Paramecium bursaria* Ehrenb., *Euplotes daidalos* Diller & Kounaris and *Euplotes patella* Ehrenb., having *Chlorella* spec. as intracellular endosymbionts, also called zoochlorellae. In foraminiferans and radiolarians, e.g., *Eucoronis* spec., other algae are endosymbionts, i.e., mainly dinoflagellates but also chrysomonads, cryptomonads, and diatoms, called zooxanthellae.

The large diversity of heterotrophic and autotrophic partners in such various holobionts mirror the trend of organisms toward assembling to holobionts as a rule rather than exception. Since the phenomenon is encountered already at the basal level of protists, we may conclude the beginnings to have occurred already during the early evolution of life.

2.1.2 Seaweeds

In the marine habitat many seaweeds are holobionts carrying microbiomes of epi-bacterial communities which can be beneficial or detrimental. Such systems have been particularly studied in the red algae *Delisea pulchra* (Greville) Montagne (Egan et al. 2013, Lachnit et al. 2011, 2016) and *Agarophyton vermiculophyllum* Ohmi (Saha and Weinberger 2019; Saha et al. 2020).

2.1.3 Zooxanthellae in Invertebrates

Many multicellular animals are well known to carry zooxanthellae as endosymbionts allowing them to photosynthesize. Among them are jellyfish, bivalve molluscs, sponges, and flatworms. The most prominent example are reef-building corals (Rosenberg et al. 2007; Zilber-Rosenberg and Rosenberg 2008). Coral reefs are built up by polyps with endosymbiotic dinoflagellates of the genus *Symbiodinium*. Entire coral reefs are giant holobiont-like systems (HLS, Sect. 5).

2.1.4 Fungi and Algae

Symbioses between fungi and algae are *Geosiphon pyriformis* (Kütz.) F. Wettst. and lichens. In these cases, the association of partners leads to the formation of characteristic morphological patterns. Therefore, the symbiotic organisms of lichens are conceivable as species. However, this kind of species interpretation is challenging, which shall be discussed in a separate Sect. 3.3 below in relation to the species concept.

2.2 Higher Plants

2.2.1 Different Spheres with Microbiomes

Life is intruding into and occupying any available and accessible space (Lüttge 2016). Therefore, it is not surprising to also observe all plant surfaces to be inhabitable by other organisms. The established systems of epiphytes form the phyllosphere, caulosphere, and rhizosphere as associated with the major organs of Higher Plants, i.e., leaves, shoot axes (including stems), and roots. Consistently, anthosphere and carposphere are encountered in flowers and fruits, respectively. These organismic associations (i.e., "spheres") in their entirety render plants holobionts. The interactions of the organisms within these spheres may remain superficial, e.g., by sharing space and resources in commensalism. However, interactions can also become intimate in determining mutual fitness, so that in such cases the term "holobiont" is obligatory. In addition, endophytes constitute a plant-internal sphere, the endosphere, within which interactions are functionally close. The microbiomes of these spheres differ by species composition due to morphologies and above or below-ground influences.

2.2.2 The Phyllosphere

A detailed review of the phyllosphere is presented by Lindow and Brandl (2003). It is estimated that on Earth 6.4×10^8 km^2 of terrestrial leaf surface is colonized by microbes. The biodiversity is tremendous as determined by viruses, bacteria, cyanobacteria, and archaea (often forming aggregates), algae, yeasts, and filamentous fungi. In addition to the microbes, often lichens and bryophytes are encountered. Occasionally even Higher Plants germinate in the phyllosphere, especially in the moist tropical forests.

Intense interaction between the various partners characterizes holobionts. Multipartite relationships between leaves and their microbiome include alteration of plant surface properties by microbial production of biosurfactants and phytohormones, such as indole-acetic acid (IAA) and cytokinins, affecting growth and development.

The microbiome can affect the plants with respect to resistance to biotic and abiotic stresses but also may house plant-pathogenic bacteria.

2.2.3 The Caulosphere, Anthosphere and Carposphere

The other aerial surfaces of plants provide different environments. Stem surfaces are often hydrophobic due to bark suberization and wax covering. They are nutrient-poor. Changes of temperature and moisture challenge and potentially limit the microbiomes. Nevertheless, we find rich life in the caulosphere, namely fungi, aerial algae often forming extended green coverages, lichens and bryophytes. Conversely, the various parts of flowers provide a rich variety of attractants for flower specific microbiomes in the anthosphere, where fungi are most frequent followed by bacteria. There are also abundant attractive compounds in the fruits of the carposphere.

2.2.4 The Rhizosphere

Among the spheres described here, the most essential one for the life of plants is the rhizosphere (Pinton et al. 2007). This is based on the different functions of bacteria in the root microbiome and the hyphae of fungi in mycorrhizae. The term “rhizosphere” has been introduced and defined in 1904 by Hiltner (Hiltner 1904) as the narrow interface between plant root surface and associated soil particles. More recently we distinguish three spheres, namely (1) the endorhizosphere of the peripheral apoplastic spaces of roots, (2) the rhizoplane directly adjacent to the roots, and (3) the exorhizosphere extending into the surrounding soil or substrate. These spheres constitute fluent transitions rather than being separated by distinct boundaries, while the extension of the rhizosphere as a whole into the soil substrate is hardly assessable. The root microbiome is housed in the narrow surface region, while the hyphae of mycorrhizae extend far into the soil.

The Root Microbiome

The complex multipartite interactions between plant roots and their microbiomes are modulated by phytohormones produced by both the plants and the bacteria (Jha 2020; Lu et al. 2021). Involved phytohormones particularly are IAA and cytokinins, but also others such as salicylic acid (Lebeis et al. 2015). The establishment and sustainment of the root microbiomes is supported by resources supplied by the plants. Supply is mediated by a large variety of root exudates, including organic acids, amino acids, proteins, sugars, and phenolics. In addition, resources originate from mucilage and sloughed-off cells of root caps and other surfaces. The bacteria enhance plant tolerance against environmental stresses, including defense against pathogens, and they promote nutrient uptake.

With respect to nutrient uptake an outstanding case is nitrogen. In the soil, bacteria affect N cycles by nitrification, assimilatory nitrate reduction, and denitrification. The input of N by microbes fixing and reducing atmospheric N_2 can quantitatively be substantial. Prominent examples are the bacterial root-nodule symbioses of Fabales with Rhizobiaceae and of various Higher Plants with Actinomycetes establishing endosymbioses within the roots. In the external rhizosphere associations with free-living N_2-fixing soil bacteria of the genera *Acetobacter, Azoarcus, Azospirillum, Azotobacter, Beijerinckia, Clostridium, Herbaspirillum* and *Paenibacillus* are remarkable (Baldani et al. 2002; Gottsberger and Silberbauer-Gottsberger 2006). Such rhizoplane and exorhizosphere associations are particularly effective for the nitrogen nutrition of grasses in tropical savannas providing from 5 up to almost 80 kg N ha^{-1} $year^{-1}$ (Medina 1987, 1993; Lüttge 2008).

Mycorrhiza

The term mycorrhiza has been introduced for the symbioses of fungal hyphae and plant roots by Frank in 1885 (Frank 1885). Basically, the hyphae mobilize water and mineral nutrients by forming extended networks in the soil as the plants provide photosynthetic products. Mycorrhiza dominates almost all plant life, namely, 80–90% of all species and 92% of the families have mycorrhizae (only missing in a few families, viz. the Brassicaceae and the Chenopodiaceae). In view of this kind of symbiosis, all mycorrhizal plants are holobionts.

Various anatomical/cytological mycorrhizae can be distinguished, mainly of ectomycorrhizal and endomycorrhizal type. Ectomycorrhizae with their tightly interwoven fungal hyphae constitute a web, coating the side roots. In the root cortex the hyphae form a so-called Hartig-net surrounding the cells. The hyphal net remains apoplastic outside the protoplasts of the root cells although in some cases hyphae may be invasive, then representing ectendomycorrhizae. Ectomycorrhiza, being realized in most trees, is the evolutionarily most recent type of mycorrhiza. In the case of endomycorrhizae, fungal hyphae form coils for exchange of minerals and metabolites within the root cells while still staying enveloped by a phagocytotic pocket. The hyphal coils may eventually be digested by the root cells. That special form of symbiosis is represented by orchid and ericoid mycorrhizae.

The most frequent form realized in 85% of the plant families is the vesicular-arbuscular mycorrhiza (VAM). In evolution it is the earliest most original form. In the root cortex the hyphae form vesicles and very characteristic haustoria resembling tiny trees, the arbuscules. The latter penetrate into the cells and can fill entire cortex cells but remain apoplastic invaginating the plasma membrane inside, thus increasing the surface for the transfer of solutes between fungi and plants. VAM was found in fossils of *Rhynia* (Psilophytopsida) and *Asteroxylon* (Lycopodiopsida). Mycorrhizae mobilizing minerals from the barren inorganic ground were a prerequisite for terrestrialization by these early cormophytic plants in the lower Devonian 420–410 × 10^6 years ago (Layer and Lüttge 2020; Lüttge 2021a).

Individual trees may possess 15 or more different fungal species as partners. Below ground these fungi grow between separate trees of the same or different species and connect them to each other. Due to the resulting integration of individual tree-holobionts, entire forests become large holobiont-like systems (Matyssek and Lüttge 2013). With this straightforward logic mycorrhizae are an illustrative example of approaching high scales of ecological organization in envisioning holobiontic associations to underlie biological self-organization in general and enable emergence of even complex living systems (Sect. 5).

2.2.5 The Endosphere

Root nodules and mycorrhizae within the rhizosphere already provided examples of how holobiontic partners become endophytes (Sect. 2.2.4). Beyond that, the plant endosphere is highly diverse. Endophytes colonize internal tissues of all plant organs. The transitions from peripheral spheres (Sects. 2.2.2, 2.2.3, and 2.2.4) into the endosphere follow gradual paths. Endophytic fungi are already found 400×10^6 years ago in the plant fossils of the Scottish Devonian Rhynie-chert (Krings et al. 2007). Extant holobionts have fungi within stems and leaves besides those in the roots (Carroll 1988). Moreover, multiple micro-environments prevail in all the various parts of plants occupied by bacterial and fungal endophytes. A review is available by Compant et al. (2021), and Harrison and Griffin (2020) present a meta-analysis as overview. Interactions occur at and across all functional levels, including both mutualism and pathogenicity.

3 Does Holobiosis Collide with the Concept of Species?

3.1 Holobionts as Units of Evolutionary Selection

Per definition holobionts are units of evolutionary selection. For Charles Darwin the unit of selection was the organism. Later it was envisaged to be the species (Gould 2002). Can we consider, however, holobionts as organisms or species? Skepticism is expressed regarding holobionts as units of selection (Madhusoodanan 2019), as they are associations of many organismic partners. In the co-evolution of the hologenome the eukaryotic genomes develop slowly, but the microbiotic genomes much more rapidly. Nevertheless, interaction occurs genetically between the co-evolving partners. Consequently, interspecific genetic variation can alter endophyte communities. In *Boechera stricta* (A. Gray) Á. Löve & D. Löve (Brassicaceae) the microbiome in leaves (but not so in roots) is under genetic control of the host (Wagner et al. 2016). The strong interactions among the species of the microbiome within plant holobionts are both competitive and cooperative.

Such kinds of inter-microbial interactions are selective in holobiont evolution (Hassani et al. 2018). Phenotypic selection analyses with the nonmycorrhizal

Brassicaceae *Brassica rapa* L. have shown that the microbial community structure affects plant phenotypic traits (Lau and Lennon 2011). In this study the microbial community was manipulated by applying microbiomes with simplified versus complex diversities of fungi and bacteria. The ecological consequences of the plant–microbe interactions implied improved plant adaptation to stress in terms of growth and fitness traits. Thus, the microbial community structure affects patterns of natural selection and influences the evolutionary process.

Therefore, although we can take holobionts as units of evolutionary selection, we cannot readily accept them as species. An extreme example of how intriguing this question is, are we humans ourselves. Are we as holobionts really individual organisms of a species? Let us first have a look at the species concept.

3.2 The Species Concept in Relation to Holobionts

The concept of "species" is not a simple one and often questioned, especially in microbiology. Perhaps, considering the fundamental theoretical problems we have with the modern species concept, we may start with Charles Linné (1707–1778) who had named and described that many plant and animal species. His concept was typological and morphological. The most concise concept, still applied by a majority of biologists, is that of Ernst Mayr (1942, 1982). It is based on sexual reproduction and therefore he termed it the "biological species-concept." Species are reproductively isolated groups of individuals that are breeding together (panmixia). The approach proves useful in practice, when species are taken as a taxonomic rank, i.e., as type of specimen in taxonomy.

However, this implies the great problem that it excludes asexual organisms. Looking at holobionts this embraces the prokaryotic partners in the microbiomes. The notion of frequent horizontal gene transfer or parasexual processes among prokaryotes is an attempt to overcome the problem. Thus, the definition of species may be based on common gene pools. A 16S rRNA gene sequence similarity above 98.7–99% stands for genomic uniqueness (Stackebrandt and Ebers 2006). Another obstacle is hybridization between adjacent species, which is not as rare an event as often assumed. This excludes Mayr's criterion of reproductive isolation from the species-concept (Dupré 2021).

Beyond that, the theoretical challenge of the species problem has led to dozens of alternative definitions (Mayden 1997; De Queiroz 2005). In addition to the morphological-type similarities already used by Linné, such definitions are also based on the specific selective occupation of defined ecological niches or on the phylogenetic relations of lineages. De Queiroz (2005) notes three central aspects of Ernst Mayr's biological species concept, namely reproductive community, ecological unit, and genetic unit. He attempts the comprehension into a general species concept, called the "metapopulation lineage" concept. Accordingly, separately evolving linages of metapopulations are ranked as species. As assessed by this author one basic question centers around how to draw the cutoff during the evolution of lineages. Nevertheless, evidently natural selection determines the limits of species.

Given the dynamics of different combinations of eukaryotes and compositions of microbiomes constituting holobionts, the species theory does not allow to regard holobionts as species. We should keep accepting the different partners within holobionts as different species. Such a view applies to – almost – all co-evolving symbioses. Notwithstanding, conspicuous exceptions exist, the most outstanding example of which are the “species” of lichens (Sect. 3.3.2). In conclusion, we may assure ourselves, therefore, that holobiont and species concepts do not intimately overlap.

3.3 **Geosiphon** *and Lichens: Symbioses, Holobionts, and Species*

3.3.1 *Geosiphon pyriformis* (Kütz.) F. Wettst

A remarkable link between the winding paths of thinking about symbiosis, holobionts, and species is *Geosiphon pyriformis* (Kluge et al. 1994, 2002), phylogenetically representing a mycorrhizal fungus species, although no mycorrhiza has been detected so far. The eukaryotic fungus forms a symbiosis, however, with the prokaryotic cyanobacterium *Nostoc punctiforme* (Kütz.) Har. This is not a lichen because it is not an ectosymbiosis like all the lichens but an endosymbiosis. In contact with the cyanobacteria the hyphae of the fungus form bladder-like structures and take up the cells of the cyanobacteria by endocytosis. The cyanobacteria perform photosynthesis inside the fungal cells. *N. punctiforme* is also free-living, so that its symbiotic lifestyle is facultative. Conversely, it is still open, as to whether the fungus may also occur as a free-living life form.

Presuming the fungus to exclusively exist in symbiosis, then the symbiotic lifestyle would represent a rather constricted fungal development. In fact, the characteristic gestalt of the hyphae with their bladders is a specific consequence of the interaction between fungus and cyanobacteria, given that bladders are formed only in response to the prokaryotic associate (Matyssek and Lüttge 2013). Thus, in view of the intimate association of fungus and cyanobacteria, it is tempting to regard *G. pyriformis* as a species. Such view is not farfetched at all, because *G. pyriformis* is well established as an extant model of the endosymbiosis that has led to the evolution of eukaryotic cells with their organelles, mitochondria, and chloroplasts (Schnepf 1966). According to the endosymbiosis theory, these organelles originated from prokaryotes taken up by the evolving eukaryotes by endocytosis and subsequent accommodation toward serving eukaryotic energetics and metabolism. In conclusion, all eukaryotic organisms can be viewed as endosymbioses, and hence, as holobionts, while we unambiguously accept them as species.

3.3.2 Lichens

Among the lichens the effects of the symbiotic associations of fungi and/or cyanobacteria and algae on gestalt are so strongly marked that a whole taxonomy with genera and species can be established. In the symbiosis the cyanobacteria or algae are photobionts providing products of photosynthesis, and the fungi are mycobionts providing water and minerals. From the association of the symbiotic partners in lichens new forms of organization emerge which can only be generated by the partners together. This applies to specific gestalt. A large variety of growth forms exists, such as fruticose with branches, filamentous with hair like filaments, foliose leaf-like, crustose with crusts, leprose powder-like and gelatinous jelly-like. Lichens appear as emerging new self-organized morphological and physiological units, which we will not hesitate to regard as integrated organisms.

Can we consider lichens as species though? This is suggested by lichen taxonomy. The gestalt of lichens is determined by the organization of the fungal filaments between which the algal or cyanobacterial cells are embedded. Propagation of lichens is mainly vegetative. The algae of lichens can exclusively propagate vegetatively. Only the fungal partner can perform sexual propagation, which is an essential feature of Mayr's definition of species (Sect. 3.2). For such reasons specific taxonomic units (classes and orders of lichens) used within a division of "lichens" are based on the phylogenetic system of the fungi (Bresinsky et al. 2008). Ascolichenes and Basidiolichenes are lichenized Ascomycetes and Basidiomycetes, respectively. In total, 400 genera and >25,000 "species" of lichens can be classified. With the convention for classification by the fungal component the binomial scientific names of "species" of lichens are identical with those of their fungi and integrated into the classification schemes of fungi.

This is formally very practical but has conceptual difficulties. The partners in lichens are from different kingdoms of organisms, viz. (cyano-)bacteria, plants (the algae), and fungi. They belong to different ancestral lineages. A given mycobiont can enter into symbioses with different photobionts, which according to the convention would all have the same species name. Notwithstanding their formal taxonomy, lichen "species" are therefore distinguished from the biological species within the kingdoms of organisms.

Relating holobionts and species we possibly join both closest to each other when taking *Geosiphon* and lichens as examples of holobionts. However, we cannot claim identity: Although lichens are particular life forms and unitary organisms as units of evolutionary selection, not necessarily the status of species can be implied. We need not be concerned that concepts of holobionts (Sect. 1) and species (Sect. 3.2) are colliding.

4 Evolution and the Heredity of Microbiome Genetic Information

Heredity is one of the central steps in Darwinian and Neo-Darwinian concepts of evolution. Doubtlessly, microbiomes can be transmitted between holobionts. In the animal kingdom this also comprises mankind, where vertical transmission leads to phenotypic variation and heritability of human traits (Funkhouser and Bordenstein 2013; Sandoval-Motta et al. 2017a, b). This includes diseases, where microbiome-manipulation opens ways for medical therapeutical interference by microbiome-engineering. Inheritance of the genetic information within the microbiome partially explains the phenomenon of "missing heritability," being observed when assessments are exclusively based on the host genome (Sandoval-Motta et al. 2017b). In the plant kingdom microbiome transmission can occur via seeds as we shall see below (Mitter et al. 2017; Berg and Raaimakers 2018; Abdelfattah et al. 2021).

We must distinguish four different ways of transmission, namely of horizontal and vertical type, each characterized by transient and, in a strict sense, permanent transmission, respectively (Abdelfattah et al. 2021). Transient transmission is superficial where parts of the microbiome are temporarily moving between individual holobionts. Transmission sensu stricto is permanently fixed. Horizontal transmission occurs at the level of co-existing holobionts. Vertical transmission is transgenerational between parents and offspring.

The role of seeds in transmitting the microbiome in plants has been studied with acorns of oak (*Quercus robur* L.) by Abdelfattah et al. (2021). They comment on the four ways of transmission. They have characterized the distribution of genera of microorganisms in parts of the seeds using fungal ITS and bacterial 16S rDNA. Microbial taxa present in embryo and pericarp are transmitted to the phyllosphere and the roots of the emerging seedlings and plants. The high fungal and bacterial diversity in seedlings proves that the persistent vertical transmission is effective heredity of the microbial part of the hologenome, i.e., the microbial genetic information is transferred to offspring. That kind of generic heredity via species composition of the holobiont has important consequences for understanding evolution. Since a holobiont is a single evolutionary unit with the close co-evolution of the associated partners, the role of the microbiome in evolution is intriguing. Host genes are inherited in the classical Mendelian way. However, evolutionary selection of holobiont units also encompasses selection covering the microbiome. Given the vertical transgenerational transmission of the microbiome, its selection acts apart from genetics. This poses a challenge on the modern synthesis of Neo-Darwinism (Sandoval-Motta et al. 2017a).

5 Large Scale Systems as Holobionts

5.1 *Below-Ground and Above-Ground Connections: With Mycorrhiza and* Cuscuta *from Individual Holobionts to Holobiont-Like-Systems (HLS)*

It was noted above that the hyphae of mycorrhizal fungi interlink all the individual trees of forests. As a result, forests become holobionts at large scale dimensions beyond the level of individual organisms (Sect. 2.2.3). Above-ground, a comparable example of such connections is given by the parasite *Cuscuta* (Matyssek and Lüttge 2013). Although spatially by far not being as extended as mycorrhizae, *Cuscuta* reflects the same principle.

Cuscuta (Convolvulaceae) is a genus of obligate holo-parasites, comprising many species worldwide (Dörr 1972; Fischer et al. 2021). From its thin and long winding stems, that have been compared to silk, it produces endophytic haustoria tapping the xylem and phloem of its host plants for all its resources, water, minerals, and organic compounds. However, this exploitation is not a one-way process but there is a dynamic exchange. A given plant of *Cuscuta* can attach itself to at least two different host plants (personal observation in a Cerrado-savanna in Brazil; Matyssek and Lüttge 2013). Birschwilks et al. (2006) have studied this phenomenon experimentally. They have taken two vascular plants, one used as a donor plant and the other one as an acceptor plant, and established between them a *Cuscuta*-bridge. Labeling with ^{14}C-sucrose or fluorescent dyes showed sugar and dye to move from the donor to the acceptor plant (both *Vicia faba* L.) via a bridge of *C. reflexa* Roxb. In *Nicotiana benthamiana* Domin even a virus-particle moved the same way.

In the above observations, holobionts comprise three partners, i.e., donor and acceptor plant and the bridge. We may assume *Cuscuta*s by vigorously overgrowing extended vegetation to produce haustoria into more than two different host plants, which illustrates the HLS concept even more vividly. By creating a bridging network above-ground that enables coupling and metabolic integration across host plants, *Cuscuta*-mediated HLSs are conspicuously analogous to those of mycorrhizae below-ground.

5.2 *The Holobiont-Like-System (HLS) Concept*

The definition of the holobiont sensu stricto (Sect. 1) comprises three essential aspects: Required are (1) a host organism, (2) associated micro-organisms, and (3) interaction between the associates. Focusing on the interaction of associated organisms pari passu without stressing a central function of hosts, i.e., moving beyond host-centered organismic associations, we attain a generic vision. Holobionts then become larger emergent and self-organized units (Lüttge 2012a, b) with functional interaction networks across all associated and integrated lower units. That vision does not only comprise holobionts sensu stricto and symbioses sensu De Bary (1879) in general, but also extends the holobiont concept to any scale of biological organization. Explicitly high ecological scales are reached, characterized by habitats, ecosystems, landscapes, biomes, and the entire biosphere, all being dominated by plants (Sect. 5.3). To characterize this conceptual advancement toward a broadened view versus the holobiont sensu stricto, the principle of holobiont-like systems (HLS) is envisioned as based on the following definition: *Emergent biological system in interaction with all its biological and abiotic components for selection in evolution* (Matyssek and Lüttge 2013; zu Castell et al. 2016; zu Castell 2019; Lüttge 2021a; Matyssek et al. 2022).

The principle of HLS is encountered as progressing hierarchically along ecological scales, starting at that of populations toward HLS Earth, i.e., the global scale. Respective HLSs are units or knots in a hierarchy of interaction networks along increasing scalar levels (Lüttge 2021b). When dwelling on the botanical aspects related to HLSs in the following section (Sect. 5.3), we must stay aware of the ecological relevance of all organisms, including microbiomes, animals in addition to plants, and the abiotic environments in each of the hierarchically increasing HLS units.

5.3 Plants in the Holobiont-Like-Systems at Higher Scalar Levels

In the case of forests, we have encountered a large extended HLS above. Independent of mycorrhizae (Sect. 5.1), the additional phenomenon termed symphysis prevails, creating root networks across forest trees. The network is established by natural grafting (intergrowth) of roots between tree individuals of the same or even different species (Bormann 1966, Aloni 2021: p 192), being in support of the holobiont principle. Like forests, most holobionts are dominated by plants. Conducive may be the sedentary lifestyle of plants, whereby they provide ecological niches to other organisms. Plants were the pioneers in terrestrialization of life on Earth (Layer and Lüttge 2020; Lüttge 2021a), shaping the terrestrial abiotic environment (Lüttge 2016). Describing HLSs with their plants at hierarchically increasing ecological scales, in each of the interacting units the knots across network hierarchies deserve attention. As pointed out above, respective hierarchies are determined by (1) populations in habitats, (2) ecosystems, (3) biomes, and ultimately (4) the entire biosphere (Matyssek and Lüttge 2013).

5.3.1 Populations

Particular HLSs are populations of clonal plants, especially of trees. Specifically in the genus of *Populus* the largest natural clones exist, e.g., *P. euphratica* Oliv.

occupying more than 4 ha in China (Bruelheide et al. 2004) and *P. tremuloides* in the USA with 42.9 ha (Grant 1993) or even 81 ha (Kemperman and Barnes 1976). Covering such large areas and extended habitats the individual trees of these clones are associated within HLSs (Matyssek and Lüttge 2013).

5.3.2 Ecosystems

Ecosystems as defined by Tansley (1935) as natural units are extraordinarily complex. The living organisms in a given ecosystem can belong to all kingdoms of life, bacteria and archaebacteria, plants, fungi, and animals. They are closely interwoven within a plethora of dynamic interactions. However, they are units in the understanding of Arthur George Tansley or, as we claim, as HLSs. "Scale" in the context of ecosystems is a matter of complexity. Spatially, scales may be small, as in the case of biofilms, although soil crusts with cyanobacteria, eukaryotic algae, fungi, lichens, and bryophytes as well as invertebrate animals can cover large areas notably in savannas and deserts (Belnap and Lange 2001). Other terrestrial and marine ecosystems can also vary considerably in their spatial extensions. Obviously, forests are ecosystems and have been used above as a prototype example for introducing into the principles of "botanical" HLSs. Forests are HLSs that attain considerable spatial extensions determining zonobiomes, as exemplified by tropical and temperate rainforests or the taiga, as highlighted below in Sect. 5.3.3.

5.3.3 Biomes

The biogeographic literature presents a certain variety of definitions and concepts of "biome." They have many similarities but contain more or less differing lists of biome types. Biomes may be considered as "groupings of ecosystems" (Whittaker 1962, 1975). Accordingly, biomes as composed of the sub-units of "ecosystems" become superior HLS units along the ecological scaling hierarchy.

Under botanical perspective, Earth can be represented by vegetation zones. The 30 vegetation zones mapped in a textbook (Bresinsky et al. 2008; Kadereit et al. 2014) give a pretty complete description of the Earth's surface. Founding surface characterization on vegetation, i.e., plants, appears justified because plants shape the environment (Lüttge 2016) as the primary producers of biomass, enabling ecological niching by other life forms. In such a way, members of the other kingdoms of life participate in biome HLSs. Therefore, we adopt a biome-concept comprising all life.

Realizing, in agreement with Heinrich Walter, that the climate is the primary factor determining expression of plant life on our planet's surface, resulting vegetation zones can be verbalized according to climate terminology. On such grounds, the 30 vegetation zones can be comprehended to 9 zonobiomes (Walter and Breckle 1984, 1991, 1999), as listed in Table 1. The account proves the delineation of zonobiomes to closely relate globally to plant life forms and climate. Notwithstanding, azonal biomes within the climate zones must not be overlooked, such as

Table 1 Zonobiomes according to Walter and Breckle (1984, 1991, 1999)

Zonobiome	Comment
Equatorial zonobiome	evergreen wet tropical forests
Tropical zonobiome	summer rain and various types of forests and savannas
Subtropical arid zonobiome	desert climate
Mediterranean zonobiome	winter rain and evergreen laurel-like hard leaf and deciduous forests
Warm temperate zonobiome	temperate rainforests
Temperate nemoral zonobiome	summer-green deciduous broadleaf forests
Arid temperate continental zonobiome	steppes and prairies, i.e., grasslands subject to frost
Cold-temperate boreal zonobiome	taiga of evergreen coniferous forests
Arctic and Antarctic zonobiome	tundra dominated by evergreen and deciduous shrub species

orobiomes determined by altitude and pedobiomes by edaphic characteristics, or extrazonal biomes enabled by specific local conditions.

5.3.4 Biosphere, HLS-Earth or Gaia

Conceptually climbing up toward the high scales of ecological organization, may one conceive HLSs such as biomes to merge into even larger HLS units? The next higher and ultimately reachable scale is the entire biosphere, comprising all life on Earth. The biosphere actually is the ultimate HLS, because the limits of the globe with its surface of 510×10^6 km^2 prevent further upscaling, if we neglect possible life on and its inter-relations between other planets of the universe (Steele et al. 2018), which at present remains science fiction. On such grounds, the biosphere as the integral of the subordinate biomes can be regarded as HLS-Earth (Matyssek et al. 2022). This hierarchically highest biospheric HLS level incorporates the lithosphere, hydrosphere, and atmosphere with the entirety of biotic and abiotic interactions. HLS-Earth conceptually conforms with the vision of "Gaia" as proposed earlier by Lovelock (1979, see also Matyssek and Lüttge 2013, Matyssek et al. 2022) as a "complex entity" involving all life on Earth. The picturesque metaphor of Gaia, the broad-breasted mystical Greek goddess Mother Earth, stands for a self-organized, in Lovelock's words a "self-regulating," supra-organism. However, the concept of HLS-Earth reaches farther in mechanistic terms (Matyssek et al. 2022).

5.4 Evolution of HLS

Evolution is a central aspect in the concept of holobionts, and clearly holobionts sensu stricto are units in Darwinian and neo-Darwinian theories (Sect. 3.1). However, for HLSs at high ecological scales, especially that of the biosphere, the question of evolution becomes intriguing and seemingly poses problems, because three fundamental attributes of neo-Darwinian evolution appear to not being realized, such as (1) mutations, (2) off-springs for heredity, and (3) competition for selection. Notwithstanding, genotype–environment interactions exist, which, e.g., contribute to the complexity of microbiome assembly in natural environments (Wagner et al. 2016). Evolutionary ecology considers ecosystem evolution (Sandoval-Motta et al. 2017a). Within such contexts, endeavors extending evolution theory beyond genome-centered neo-Darwinism (Kimura 1983; Carroll 2000; Scherer 2017) open new explanatory perspectives, which in being pursued pose a formidable theoretical challenge for understanding HLS evolution. We argue and give evidence in a separate article in this volume of Progress in Botany (Matyssek et al. 2022) that HLSs irrespective of ecological scale, i.e., including HLS-Earth, do undergo evolution. The other article expounds science-historical, theoretical, and provable grounds of scale-invariant (generic) mechanisms of HLS evolution, being summarized here in a nutshell:

Competition prevails in HLSs, both in direct concurrence and between their subsystems, given the need to perpetually adapt to dynamic cause-effect relationships as imposed by the selective pressure of the ever-changing external and internal, biotic and abiotic conditions. The requirement of permanent adaptation to ensure persistence underlines that immediate competition in a conventional sense is not a mandatory prerequisite for HLS evolution. An example is HLS-Earth in the absence of competing HLSs at the same ecological scale, where evolutionary progress is apparent throughout Earth's history since the beginnings of life. Planetary ageing sets the stage for HLS-Earth evolution, progressively shaping appearance and properties of the biosphere over geological times, although the persistence of life and its fitness have been challenged at least five times through global waves of species mass extinction. In adaptive responses species diversity and capacities of ecological niching have progressed perpetually, giving proof of evolutionary advancement even at the scale of solitary HLS-Earth. The underlying functional core is sequential selection as innate part of adaptive cycling, which continuously proceeds through four oscillatory phases of renewal and consolidation: the creativity phases of resource release and reorganization upon HLS stages of decline, and the consolidation phases of resource exploitation and conservation during stages of HLS renewal. Each turn in cycling mirrors evolutionary advancement under varying environmental impacts.

6 Conclusion

Holobionts and holobiont-like-systems are ubiquitous. They are the universal associations of living organisms of the differing kingdoms of life, covering all scales of organization from protists to the entire biosphere. That principle is corroborated by an overview of botanical examples, notwithstanding that each naturalist or plant biologist may discover holobiont-like properties of her or his own objects of observation and study. The interactions of the living partners are highly dynamic, so that holobiont is not just a state, but holobiosis is a process. Holobiont-like principles dominate evolution, and holobiosis directs our reflections toward new extended concepts of evolution theory.

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Evolution of Holobiont-Like Systems: From Individual to Composed Ecological and Global Units

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> It's the song, not the singer
> (W. Ford Doolittle & A. Booth)

Contents

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Abstract Higher Plants or Animals with microbial companions constitute holobionts, being spatiotemporal interaction networks as (co-)evolutionary selection units. As intrinsic interaction patterns also prevail at hierarchically different scales of ecological organization, organismic associations scale-invariantly represent holobiont-like systems (HLSs). This essay explores how high-ranking HLSs (ecosystems, biosphere) do evolve, in particular, under the conventionally gene-centric view at the Darwinian principles of mutation, heredity, and selection.

Re-visiting evolution theory, shortcomings by DNA-restricted interpretations impede perceptions of HLS evolution, notably, at hierarchically high ecological scales. Any HLS is discernable as a selection unit, however, through considering genericness by widening DNA-encoded to structurally and functionally stored information upon historical contingencies in "EvoDevoEco" processes, relating evolution, development, and ecology. On such grounds, HLS evolution proceeds through adaptive cycling and sequential selection, examining predictability versus stochasticity of aggregated information for adaptiveness to environmental variation. Each turn mirroring evolutionary advancement, cycling does not require resource-related competition as evolutionary driver. However, selection is reflected in competition in a sense of facing the challenge to maintain internal stability upon environmental changes. Such challenge becomes evident also in biospheric HLS Earth evolution through traded contingency effects upon manifold biogeochemical impacts. Although repeatedly inciting global species mass extinctions, such were pulse generators towards niches diversification and ecological complexity, owed to recurrent valuing scrutiny of sequential selection. Abandoning gene-centric perspectives, HLS evolution is borne by oscillating phases of consolidation versus renewal.

Keywords Adaptive cycles, Biosphere, Ecosystems, Evolution, Life criteria, Neo-Darwinian revision, Panarchy, Sequential selection

1 Introduction

Technological advances such as next generation sequencing have led to an explosion of work unraveling interactions between Higher Organisms and their microbial companions. Considering a host organism and its associated microorganisms as a functional entity has originated the notion of the holobiont (Meyer-Abich 1943; Margulis 1998; Rosenberg et al. 2007; Baedke et al. 2019). Being an entity of evolution, holobiont is basically defined as: "*A host organism in interaction with all associated microorganisms as a unit for selection in evolution*" (Zilber-Rosenberg and Rosenberg 2008). Hence, the individual host with its microorganisms is denoted as scaling unit. With holobiont research gaining substantial scientific popularity (see, e.g., Simon et al. 2009, 2019, Faure et al. 2018), the definition can be extended from hosting individuals with microorganisms to associations of organisms in general, i.e., sensu functional interactions between "*all associated organisms as a unit for selection in evolution*" (Matyssek and Lüttge 2013). Such associations are symbioses sensu stricto according to De Bary (1879), being mutualistic or parasitic in a broadest sense, even without being explicitly beneficial or detrimental to involved agents or requiring a centrally acting host (Matyssek and Lüttge 2013). The coercive further step is to extend the scope to next higher ecological scales such as populations, ecosystems, and biomes, ultimately reaching the entire biosphere, i.e., "ecosystem Earth" (Matyssek and Lüttge 2013; zu Castell et al. 2019; Lüttge 2020) or Gaia sensu Lovelock (1979, 2009). Regarding all such scales including that of the holobiont sensu stricto, the term "holobiont-like system" (HLS) is applicable, and consistently definable as "*emergent biological system in interaction with all its biological components for selection in evolution*" (Matyssek and Lüttge 2013; zu Castell et al. 2016; Lüttge 2020). The essence of the HLS definition even holds for subordinate scales relative to that of the holobiont sensu stricto, i.e., compartments within individual organisms such as the cells with their prokaryotic endosymbionts. Such "lower scales" within the HLS concept have been outlined already in detail by Matyssek and Lüttge (2013) and zu Castell et al. (2016). However, the perspective towards "high" ecological HLS scales, such as ecosystems and biosphere, still requires focused examination regarding mechanisms of selection and evolution (zu Castell et al. 2019). To that end, the HLS definition allows for leaving the scales of the holobiont sensu stricto with its individuals and their internal compartments by stressing the eminent functional role of ecological interactions across involved agents, i.e., holobiont-characteristic relations, in general and at any ecological scale (Sects. 2.1 and 2.2).

As evolution is a fundamental aspect in holobiont definition, one key issue is about how to conceptually arrive at holobionts and HLSs as units of selection (Sects. 2.3 and 2.5). The issue is challenging, as evolution actually means co-evolution at any HLS scale (Sect. 2.4). The aim of the present essay is to clarify this issue. For Charles Darwin the unit of selection was the individual organism, later extended by Ernst Mayr (1940, 1942) to the scope of species. However, at scales of ecosystems and above the raised issue of evolutionary units becomes intriguing. The challenge is bound, therefore, to face strong current moves of abandoning straightforward ideas about selection as inevitably interwoven with the genome-centered dogma of evolution. We will pursue, instead, and according to a second synthesis, an extended generic concept of continuous, comprehensive, and dynamically co-acting evolution processes. Grounds are prepared for promoting still underdeveloped aspects of the evolution theory by exemplifying principles of HLS evolution.

In such a sense, this essay frames within the Extended Evolutionary Theory (Carroll 2000; Pigliucci 2008; Danchin et al. 2011; Mesoudi et al. 2013). The extent is to be examined, to which patterns of continuous adaptive cycles in dynamic interplay with the environment (zu Castell et al. 2016), including the global scale (Sect. 3.1), may provide keys to generically understanding evolutionary progress (Sects. 3.2 and 3.4). The way such progress arises from evolution/development/ecology phenomena is explored under EvoDevoEco perspectives (Sect. 3.3). Pursuing such a rationale of continuous sequential adaptations (Sects. 3.2 and 3.4), we will approach our ultimate aim in this essay of understanding HLS evolution (Sect. 3.4) along ecologically high scales up to HLS Earth (Sects. 4.1 and 4.2.). In which ways is evolution identifiable at high HLS scales, of necessity, here, beyond strictly genome-centered scopes and conventional views of competition?

2 The Essence of Holobionts and Holobiont-Like Systems (HLSs)

On theoretical grounds, the holobiont sensu stricto requests one agent as host in the center of an interaction network of involved associates (Fig. 1a, zu Castell et al. 2016). Releasing holobionts from that centristic view led to the concept of holobiont-like systems (HLSs), being defined as an association of equitable biological agents subject to (co-)evolution in a generic sense (Fig. 1b, zu Castell et al. 2016). Both holobionts and HLSs, alike any organismic system or entity, emerge from interaction networks with features of feedback-controlled self-organization and regulation of the energy and matter exchange with the environment (Fig. 1a, b). Stages of consolidation arise that reach beyond scopes to be expected on the grounds of random interactions. Only self-organization allows us, as observers, to distinguish biological systems (such as holobionts and HLSs) from abiotic randomness, being aware of fluently circumstantial transitions. Under such premises, we will open our exploration by re-capitulating basic essentials of HLSs.

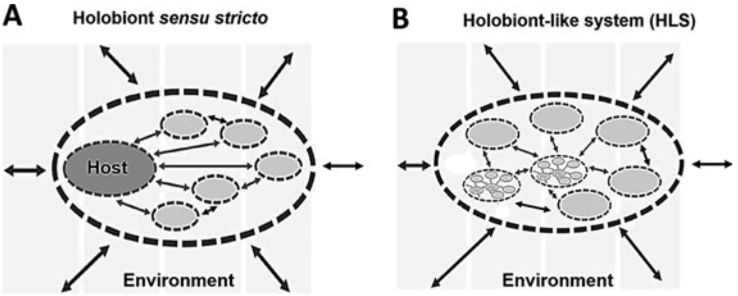

Fig. 1 Holobiont sensu stricto (Hss, **a**) and holobiont-like system (HLS, **b**). (**a**) Hss consist of a central host (e.g., Higher Plant or Animal; dark gray ellipse) and associated microorganisms (plus other organisms, when conceptually extended; light-gray ellipses), which altogether form an interaction network among each other and with the fluctuating environment (double-arrows). Broken lines denote Hss as a whole, host and associated organisms as open systems each, allowing exchange of resources (energy, matter) and intrinsically transferred information. In such a way, Hss emerge as co-evolutionary unities, which jointly adapt to environmental stress, including such by co-occurring other Hss. (**b**) HLSs, if at ecosystem level, comprise Hss or, in the case of HLS Earth (i.e., the biosphere) subordinate ecosystem-level HLSs. Contrasting with Hss, HLSs do not rely on a central host, rather subordinate unities are equitable within the interaction network. Two of the ellipses exemplify the recursive internal network structure of HLSs across subordinate ecological scales. In consistency with Hss, HLSs are open systems (broken lines) and functionally constitute co-evolutionarily adaptive unities. Comparing (**a**) with (**b**), Hss turn out as special cases of HLSs (adapted from zu Castell et al. 2016)

2.1 Ecological Settings and Scales of HLSs

Recalling the ecological and evolutionary significance of holobionts, these rather than individual organisms interact within ecosystems (Fig. 1a, b) and promote, as the actual drivers of ecological niche formation, fitness and complexity within and diversity between ecosystems. The latter can be conceived, therefore, as interaction networks between holobionts and with prevalent abiotic site conditions and neighboring ecosystems (Fig. 1b), altogether enabling for varying degrees of ecosystem self-containment and persistence. Such networks, however, also exhibit a certain degree of retention, allowing evolution to accumulate information over time. Such a view of interacting agents is in line with definitions of ecology and ecosystems by Haeckel (1866), Tansley (1935) or Ellenberg et al. (1986). Indeed, we will demonstrate below that ecosystems fulfill the requirements of being HLSs. Consistently, HLSs are encounterable at even ecologically higher scales of landscapes, zonobiomes or even the entire biosphere, as in all such cases systems are borne by intrinsically holobiont-like interacting sub-systems, being part of between-scale and within-scale interaction networks (Fig. 1b). Consequently, ecosystems are borne by subordinate holobionts, landscapes and zonobiomes by their ecosystems, and the biosphere as a whole by the global entity of ecosystems. All these scales represent HLSs each, as ultimately does the biosphere as HLS Earth. Consequently, also holobionts sensu stricto are HLSs, with their down-scaled interaction networks between subordinate organismic agents, their associates each and the biotic and abiotic environment (Matyssek and Lüttge 2013; zu Castell et al. 2016). Following zu Castell et al. (2019), however, it is the ecologically upwards directed scaling perspective pursued here in this essay.

For live systems to evolve, three interacting forces must be postulated in a Darwinian sense, namely, mutation (generation of novel information through random variation), heredity (transmission of information), and selection (enabling persistence of consolidating information). In HLSs, these forces are each encountered through the generic qualities, i.e., characteristics of universal features to a rather broad extent beyond what is solely covered by DNA and genetics. Such extended qualities are, beyond the DNA-level stored information of associated organisms, HLS-level species abundances, above and belowground structure with inherent functionalities, and geochemical transformations (i.e., of the physicochemical earthly environment), which altogether, through the entirety of overarching interactions, act as informational carriers. Hence, randomly occurring variations within the full available range of HLS-intrinsic information represent generic mutation, being the driving force of HLS adaptability to environmentally induced stress. Stress is neutrally valued here, both in a strictly physical sense, as any environmental stimulus (i.e., beyond any potential benefit or adversity), and as integral biotic and abiotic impact, being a unique characteristic of live Earth (i.e., its biogeochemistry). Such generic informational variation leaves traces in the interaction network and is stored through various mechanisms, enabling the network's plasticity, so that informational transformations are forwarded over time, accordingly, by generic heredity.

Generic selection, which other than generic mutation and heredity is not system-immanent, but an autonomous process, acts at HLS level in that generated and forwarded fluctuations may gradually elicit alteration of HLS properties. Those being ecologically conducive under the current environmental conditions become sharpened through fostered adaptation. Others fade out, if they do not promote ecological stability. HLS properties are also shaped by intruding information from neighboring HLSs as a result of overarching differential selection. Thus, selection emerges as an autonomous process through continuous action of various HLSs within a given environment. In such ways, ecosystem-level HLSs may evolve into altered or novel HLS types conveying persistence, or may fail by ultimate collapse or replacement.

Such progressions mirror selection and evolution at HLS scale. Retrospectively, they consolidate ecologically advantageous HLS expressions due to enhanced likeliness of persistence, relative to others that passively fade out. Selection in generic terms does not even require competition between HLSs, as also intrinsic biogeochemical changes challenge HLS performance, either towards compliance with novel ecological settings or disappearance as two alternatives. The conclusion also holds for HLS Earth as the integral global interaction network across ecosystems, lacking competition of hierarchically equivalent HLSs. HLS-level adaptations, irrespective of being attained in the presence or absence of competition, but marking the transition from full-range informational variation towards persistence as the outcome of selection, signalize evolutionary progress towards system consolidation. As arising from the generic qualities of mutation and heredity, and hence, inherently from selection, also adaptability acquires generic qualities. HLSs evidently evolve through informational turnover as borne by multilevel interaction, selective shaping and emerging informational storage and transfer, by inherently obeying functional mechanisms.

The HLS concept faces risks of being reduced to a narrowed explanatory basis in evolution theory restricted to frequency assessments of genetic alleles within genotype populations. In view of the considerations outlined above, such risk is not substantiated, if generically widening informational turnover beyond strictly DNA-related scopes. Transferable information subject to selection is anchored also in epigenetic principles, HLS-associated population and species diversities and abundances, new arrivals and drop-outs along with other structural and functional above and belowground HLS properties, including geochemical features. In particular, the notion of fitness, if solely conceived as number of offspring, would lead to a dead conceptual end. In addition, fitness is typically assigned a posteriori to underlying genotype–phenotype associations. Concluding a phenotype to be fitter than others, rests on conceiving the corresponding genotype to acquire a larger share within the genotype distribution (zu Castell et al. 2019). Although being crucial in shaping genotypes, the notion of fitness conventionally ignores, partially at least, the environmental impact. Rather, "*the flexible environment must also be included along with the flexible organism … The unit of survival is a flexible organism-in-its-environment*" (Bateson 1972, p. 451). The statement of survival as mediated through flexible organism-in-environment embedment forwards the core of the HLS concept, supporting the ecological dimension of fitness. That dimension is to be expressed by the capacity of creating and using ecological niches and, hence, enhancing the survival chance of offspring and its adaptability, and ultimately, HLS evolution. Fitness at HLS level, as addressed here at scales above holobionts, also attains, therefore, generic qualities. Progressive niche diversification as an aspect of fitness warrants HLS consolidation and persistence.

This is what ecology has worked out. Focusing on interactions, the theory of ecological orientators (Bossel 1998), e.g., as promoting diversity in ecological niching, embraces the above-mentioned idea that any acting biological system leaves traces on its environment. Such traces reflect the inner organization of the system itself. Ecological niches (Hutchinson 1957) upon leaving their traces provide a particular example. The biodiversity-related niches differentiation theory (Gatti 2011) shows the mere presence of species to augment niching capacities to be explored and used by other species. Thus, species absence or presence strongly shapes species communities and, hence, HLS structure, including genetic diversity at meta-community levels. Striving for a unifying evolution theory requires an integral view from agents to their interaction networks in view of HLS-relevant fitness and in defining actual selection units (see Bateson 1972; Popper 1986, 2013; Jablonka and Lamb 2014; Doolittle and Inkpen 2018).

2.2 Consistency of HLSs with Criteria of Life

Ascribing features to HLSs such as mutation, heredity, adaptability and memory, which altogether are pre-requisites of and indispensable to life, we may first ask, as to whether HLS performances are functionally consistent with life expressions as commonly expected by us, as observers, from individual organisms.

We have no simple and straightforward definition of life and must work with listing its dominant features. Darwinian evolution does not tell us much about the basic properties of life. Darwinism is not conceived to explain the origin of life. Theories of evolution unfold the temporal development of existing life. However, we can identify features of life in HLSs by applying the *PICERAS framework*. Seven features are claimed, altogether indispensable to identifying living systems. The acronym was coined by Koshland (2002), linking the initial letters of the postulated life criteria, which as compared with previous approaches are more generically defined to become applicable also to biological scales other than that of individual organisms. The criteria are: *P* = *program, I* = *improvisation, C* = *compartmentalization, E* = *energy, R* = *regeneration, A* = *adaptability,* and *S* = *seclusion.*

Accordingly, *Program* expresses life to obey informational settings, e.g., encoded by DNA, epigenetic modifications or structural and functional properties in species communities as resulting from contingencies in co-evolutionary developments. *Improvisation* stresses the capacity of life to react to unforeseen environmental changes through transitional reversible acclimation or permanent irreversible adaptation. *Compartmentalization* denotes living systems to be recurrently structured into sub-modules, while possessing boundaries with the environment. *Energy* underlines the openness of living systems to energy and matter exchanges with the environment, while internally creating structural and functional order by means of self-organization and regulation versus externally augmenting entropy. *Regeneration* denotes transformation of energy by means of catalytic compounds enabling functional maintenance and reproduction, i.e., counteracting increase in internal entropy, as based on generically encoded system-level information. *Adaptability*, in supplementing improvisation, reveals plasticity of living systems in structure and function upon environmental impact and, hence, resilience. And *seclusion* denotes living systems (although being open) to counteract net loss of resources crucial for functionality. Evidently, PICERAS offers a concept for exploring consistencies in life expression across different scales of ecological organization.

As to be concluded, life depends, in essence, on capacities of sustaining adaptability, as the multi-level interactions (including geochemical settings) perpetually incite environmental variations, requiring means of adequate responsiveness. Some of the incited and/or impacting changes are predictable (e.g., seasonal and daily cycles), while others are not (e.g., mega-volcanic eruptions, cosmic impacts). Persistence requires living systems, therefore, to maintain capacities of acclimatory and adaptive responsiveness to overcome environmental challenges, in particular, such which are unpredictable. Under latter conditions, it is not necessarily the fittest system or, more generally, such resulting from gradual long-term evolution, but the “luckiest” one, adopting chances of survival under spontaneously arising novel scenarios. Latter systems are coincidentally able to cope with abrupt adversity and newly initiate adaptive radiation, either through re-conquering abandoned ecological niches or opening novel, unprecedented ones. It is apparent that HLSs thoroughly comply with the PICERAS principles as does any “conventionally” distinguishable organism. Dealing with continuous environmental change, HLSs need to cope with the complexity of environmental impacts they have to deal with while maintaining internal stability. Compartmentalization breaks down complexity and allows the HLS to balance internal variation and retention. Thus, compartmentalization naturally emerges as a consequence of continuing adaptation.

Given that conclusion, HSL-internal complexity in structure and function, as emerging from self-organization and regulation, is a general feature and pre-requisite of life (Souza and Lüttge 2015), in distinction from “non-life.” Only regulatory control allows for shaping ordered complexity (decrease of internal entropy) at the expense of externally fostered (unordered) entropy. However, there is no need for control to be realized by an external function. Self-organization allows for sophisticated control patterns to emerge without dedicated guidance. HLS-internal organization and complexity may be supported by external influences, although there is no rational argument to conclude that these are solely causative for the outcomes encountered today (refuting metaphysical allusions here to an active “creator” or “intelligent designer”). Complexity in the multi-level interactions between HLS components (agents) is realized by the internally arising meta-stable flux equilibria, but tends to collapse without continued net uptake of work-supportive energy. Such energy turnover blurs boundaries to the environment, so that HLS extensions and confinements somewhat depend on the judgment, i.e., research focus, of observers (zu Castell and Ernst 2012). With space as environment, the confinement of HLS Earth is more distinct.

2.3 History of Evolution Theory Towards HLS Evolution

With the aim of this essay to understand HLS evolution, including that of HLS Earth, it is important to explore the history of evolution theory in terms of capacities for advancement. On the grounds of the thoroughly affirmed life criteria of HLSs, we recognize the urge to self-stabilize as the driver of evolutionary advancement. Thus, evolution progresses randomly in the absence of directed evolutionary progress, although mediated through autonomous selection from variation in adaptability. Some historical retrospective may elucidate the beginnings of the debate:

In the middle of the nineteenth century Darwin's thinking incorporated geology, bio-geography and changes of organisms with knowledge about fossils and extinctions, environment and ecology, breeding and artificial selection. His theory was a focused, then narrowed view on selection. In his book *On the Origin of Species*, Darwin (1859) built his theory of evolution on two pillars: (1) descent of individuals with modification and (2) natural selection. The historical truth demands for also crediting Alfred Russel Wallace, concluding species to evolve by adapting to their environment. Knowing Darwin to work in the same scientific field, Wallace contacted him already in 1858, which marks the beginning of collaboration and joint publishing on evidences about natural selection and evolution (Darwin and Wallace 1858). Hence, Wallace's contribution to evolution theory at that time must be valued substantial, although mostly being overlooked. Within that field of research, it was William Paley who gave a remarkable overview of adaptation, using examples for justifying the existence of an intelligent designer. Although Darwin provided a scientific explanation of the plethora of well-adapted designs, making the role of an intelligent designer obsolete, Paley pointed out many adaptations to perfectly mirror the environment respective organisms live in (cf. Goodwin 2001). In parallel, a lively discussion on inheritance processes prevailed at the time of Darwin's above-mentioned book, as Lamarck's transformation theory of evolution represented a dominating viewpoint. Darwin's merit was introducing the principle of natural selection as driving force to the interplay between adaptation and inheritance. Focusing on competition for resources within that interplay, Darwin rated evolution to reflect the pathway of survival.

The component of survival within Darwin's theory of evolution was inspired by Thomas Malthus' book *An Essay on the Principle of Population*. Reflecting a socially adopted paradigm at that time, Malthus outlined the relation between the principle of exponential population growth until resources eventually become limiting. It is important to be aware of such historical roots of Darwin's theory, as they show an inherent economic principle right from the beginning, in terms of correctly recognizing ecological cost/benefit balances to decide about individual evolutionary success. In contrast, a system-oriented interpretation of evolutionary development does not arrive at the same conclusion, according to Bateson (1972, p. 450f): "*It is now empirically clear that Darwinian evolutionary theory contained a very great error in its identification of the unit of survival under natural selection. The unit which was believed to be crucial and around which the theory was set up was either the breeding individual or the family line or the subspecies or some similar homogeneous set of conspecifics. Now I suggest that the last hundred years have demonstrated empirically that if an organism or aggregate of organisms sets to work with a focus on its own survival and thinks that that is the way to select its adaptive moves, its 'progress' ends up with a destroyed environment. If the organism ends up destroying its environment, it has in fact destroyed itself.*" Bateson emphasizes the perspective that evolution works on groups of organisms rather than individual ones (see below for units of selection, Sects. 2.4 and 2.5). Concerning the driver of evolutionary change, Bateson concludes that an economic principle, i.e., maximization of resources, as sole driver of evolutionary adaptation would eventually lead to unsustainable exploitation of resources and finally deterioration of the environmental basis. Therefore, it is not convincing that evolution builds on an economic principle. Rather, the economic principle has to be replaced by one of maintaining adaptability. Maximization of resources may then be one possibility in realizing adaptability. Indeed, proper r-strategists implement such a functional strategy, exploiting environmental resources in consequence of their exponentially progressive population growth (according to growth rate "r"), until decimated, ultimately, by population collapse upon resource exhaustion. However, r-strategists rely on other, less exploitive strategies to co-occur in their natural environment.

Thirty years after the release of *Origin of Species* Weismann refined Darwin's concept in a gene-centric manner. The introduced Weismann barrier between soma and germ line advocates for genetically determined pathways of evolutionary advancement. Thereby, foundations were laid accentuating the gene as carrier of information within the theory of evolution. The corresponding theory of heredity consequently concentrated on the effects of mutation and variability, establishing genetics as explanatory basis of evolution. During the 1930s to 1940s, extending knowledge about paleontology, comparative anatomy and morphology, population genetics and speciation, developmental physiology and cell biology led to speaking about "modern" or "great synthesis" (Mayr and Provine 1980; Gould 2002), although selection as the central evolutionary process was hardly reconsidered. Currently, in the twenty-first century, it is argued that advancement of the theory of evolution is needed towards an "extended synthesis" (Carroll 2000; Scherer 2017).

Following Popper (1986, 2013), Darwinism is completely reducible to the two postulates that (i) variability of the genome is a genuinely random process which in principle is independent of the activity and individual preferences of the organism, and (ii) the necessity to survive within an environment, which also includes other acting organisms. Both postulates illustrate a passive role of the organism with respect to its ability to adapt. Popper contrasts this passive view of Darwinism with an active perspective. Activity of the organisms is the only element in evolution which is capable of creativity. Organisms needed to evolve by continually adapting to perpetually changing environmental conditions, and upon becoming prepared for survival, adaptation implies the ability to act (see Popper 1986). Thus, the classical, neo-Darwinian view of evolution lacks the readiness to conceive other mechanisms of information transfer such as embryonic development, stigmergy, or symbiosis as prominent drivers of evolution, implying units of selection to influence their evolution through exerting an active role within biotic–abiotic interaction networks.

The latter perception mediates to theory building in ecology, emphasizing interaction sensu activity of organisms as driver of ecosystem development. Under such a premise, concepts of ecological succession and niche building are abstracted from organisms as exerting functional ecosystem services. A fundamental insight of modern ecosystem theory is that ecosystem functionality can be provided by various species, which may substitute each other in occupying same or similar ecological niches. While such insights fostered the understanding of ecosystem functioning and, as a consequence, incited holistic, i.e., systemic approaches in perceiving ecology (Odum 1983), the aspect of inheritance as staying tied to the genome failed to become fundamental in ecosystem thinking.

However, ecological features as expressed by Zipf's Law (see Newman 2005) bring historical contingency back into focus. Originally referring to linguistics and statistical use of words, in more general terms, the frequency of any entity is predicted to be *inversely proportional* to its frequency ranking. In extension to ecology, evolutionary development leads to distribution patterns of a majority of rare, but only few predominant species (Corominas-Murtra et al. 2013). Such patterns typically emerge from processes combining informational conservation with stochasticity (Jacob 1977), which evolution is a prominent example of. In ecological terms, denoted is the higher likelihood of new incomers to establish interactions with dominant species as compared with the majority of rather rare species. Thus, resulting interaction patterns reflect historical development. In contrast to genes as informational carriers, extra-genetical historical information is at risk of getting eventually lost. However, within interaction networks that show strong local connectivity, interaction patterns can well be conserved beyond genetical scales over several generations of the interacting partners. Insights of such kind have lighted attention into microbiome research (Turner et al. 2013; Knoop et al. 2018; Moeller et al. 2018; Liu et al. 2019). Also, the role of interaction patterns in long-term development of ecosystems is appreciated both in theoretical work and ecosystem management. Examples are given by the megaherbivore hypothesis (Vera 2000) or minimal invasive strategies for ecosystem restoration (Wilson 1994). Such views on ecosystems are relevant within the scope of this essay, as they are ratable as HLSs (see above).

Returning to the question about drivers of the evolutionary process, Popper's concept of active Darwinism, as detailed above, emphasizes interactions rather than randomness to underlie creativity. Notwithstanding, interactions of one agent upon conceived environmental randomness provide a source of random change from the point of view of other agents within the same environment. Thus, interaction and randomness entail each other. Interactions convey environmental impacts on agents, as interaction patterns result from and promote randomness. The duality of complex systems' characteristics and properties of corresponding environments reflects information transfer and constitutes the key idea of the orientator theory (Bossel 1998). Thinking further along these lines, action provides opportunities for new (re-)action. The biodiversity-related niches differentiation theory (Gatti 2011) states the number of ecological niches in ecosystems to depend on that of prevalent species. Thus, enhancing diversity allows for more niches to be shaped and occupied by further organisms. Therefore, adaptation of species also reflects other species to be active in the environment. On such grounds, evolutionary development becomes a process of creating environmental opportunities and making use of them, which supports the view of integrated systems, such as HLSs at high ecological scales, to be subject also to evolution. In pronounced contrast, under gene-centric perspective evolutionary fitness is a property that arises from genotype–phenotype relationships, being a narrowed premise within ecological contexts. Evolutionary outcomes are assessable in such a case only a posteriori, disregarding the concurrent adaptability-shaping dynamics of prevalent environmental interaction networks to major extents. Resulting concepts are skewed, therefore, while opening the debate on the "proper" units of selection (Levontin 1970; Sober and Wilson 2011).

Adopting the widened perspective accentuating interaction dynamics enables understanding of evolution through species community-intrinsic cooperation. There are good reasons and ample motivation for such a widened perspective. The theory of endosymbiosis explains evolutionary development of eukaryotic cells as resulting from a symbiotic relationship of an Archaean ancestor with a photosynthetic cyanobacterium (Gray et al. 1999). Likewise, speciation is much more conclusive to be explained through cooperation (e.g., Brucker and Bordenstein 2012), irrespective of being supportive or adverse, rather than resulting from small-scale mutations and random change in the genome. Hence, co-evolution becomes apparent as the underlying principle of speciation (see below Sect. 3.1) in strong additional support of HLS evolution.

2.4 Units of Darwinian Selection

Holobiont research has gained substantial popularity in the scientific community (e.g., Simon et al. 2009, 2019; Faure et al. 2018). The holobiont concept and the associated hologenome theory of evolution (Zilber-Rosenberg and Rosenberg 2008) constitute an appealing combination of ideas about evolution and ecosystem theories. The initiated discussion reflects the unsettled challenge of evolutionary theory in assessing the proper selection unit. Suggested solutions range from pure gene-centrism as proposed by Dawkin's "selfish gene" (Dawkins 1976) to the idea of multilevel selection (Wilson and Sober 1998). Active Darwinism re-focuses, however, on interactions amongst agents. Consequently, changes in the community of participating agents coercively constitute alterations in the associated interaction patterns. New light is cast, therefore, on the question about proper selection units (see Levontin 1970).

Selection acts in changing the interactions through affecting involved agents. Since interactions are relevant on all levels of biological organization, the quest for the seemingly best level of selection (Wilson 1975) is misleading. Doolittle is using the metaphor of a song being performed by singers (see maxim cited in the essay's heading). Indeed, it is the song and not the singer which matters for evolution to unfold (cf. Doolittle and Booth 2017; Doolittle and Inkpen 2018), sensu the evolutionary information per se in the long term being important rather than the short-lived carrier of information. Freeing ourselves from pre-defined levels of biological organization, we need to understand evolution as an informational concept. Nevertheless, information per se stays ineffective as long as being conceptually decoupled from agents and their incited interactions, where it arises from. Hence, information is to be seen as part of an inextricably interrelated integral spanned across involved agents and their interactions.

In its most general sense, evolution is a process of alternating steps of variation and selection (Dennett 1995; Heylighen 1999; Mayfield 2013). As we will see later (Sect. 3.2) such alternating phases naturally arise from continuous adaptation. Hereby, evolutionarily new prototypes successively replace existing ones. Being exposed to activity by other agents, such prototypes enfold their evolutionary potential in shaping their own network of interactions. Different variants reflect differential success in their consolidation process, equating such variants, e.g., as holobionts or, more generally, HLSs. Progressive consolidation during prototype (or HLS) evolution over space or time is commonly interpreted as advancing adaptation. In order for improving adaptability to emerge from such a spatiotemporally based consolidation process, newly generated variation must be heritable at least in parts.

Evolution neither pursues ultimate goals nor pre-designed directions. Rather, it is a tinkering process (Jacob 1977) resulting from the requirement to pass on information as bound to agents and their interactions over time (generic heredity), while being forced to permanently respond to the environment. Agent-driven interaction while sensing the environment is to be seen as an informational accumulation process, which if continuing over time constitutes some form of heredity towards further informational aggregation and compaction. As all involved agents are jointly subjected to this aggregation process while sensing the environment, adaptability represents the collective outcome from self-organization towards emerging structural and functional regularities. The agents and their HLS consolidate in such a way, regarding their inherent functionalities each, through advancing adaptability, so that responsiveness is improved to environmental uncertainties. Still, such outcomes solely arise from trial-and-error advancements.

The phenomenon of structural and functional patterns to emerge from interaction results from self-organization. Any living system is self-organizing, as exhibiting and implying autonomous adaptability to changes in the environment, also holding, therefore, for HLSs. Key in supporting autonomous adaptability are reproducing capacities, consolidating more reliably than other assemblies of interaction networks. "*The favoured route to produce more life is by reproduction of existing life*" (Hoelzer et al. 2006, p. 1790). Hereby, consolidation and variation are complementary. While variation increases differentiation, selection as resulting from fluctuating states in consolidation reduces variability. The evolutionary process copes with that perpetual conflict by embedment in a sequential pattern. Thus, evolution relies on that kind of conflict as an impulse generator, triggering alternating cycles of variation and selection. In classical neo-Darwinian thinking, such impulses are set by subsequent generations. Replacing a biological individual with a system of interacting agents, such as constituted by HLSs, individual life cycles no longer are the impulse generators. Rather, according to Doolittle, the informational song is sung at HLS level in the long term by various means of generic heredity, independent of exchangeable short-lived singers sensu merely genetically confined agents. As a consequence, it is not the individual organism per se, sensu Darwin, to be seen as the target of selection. Rather, it is the functionally tied association of agents with their interactions as the co-evolutionarily relevant unit, which create the informational stage for selection to get active.

Evolving biological systems are characterized by the complementary effects of random change (creativity) and inherited degrees of stability (predictability), so that evolutionary progress is to be seen as a trial-and-error process that maintains system viability. In a Darwinian sense, evolution implies three forces to interact, namely, mutation (i.e., generation of novel information through random variation), heredity (transmission of information), and selection (enabling persistence of at least marginally beneficial information). This is why precisely these three forces have been included in the definition of the HLS (zu Castell et al. 2016). Selection as enabled through random variation is tightly linked to HLS adaptability to stress, while outcomes need to be storable through HLS plasticity as some kind of informational memory. Starting from such theoretical background, we must now ask concretely about the units of evolution in view of HLSs, up to HLS Earth.

2.5 HLSs as Units of Selection

For Charles Darwin the unit of evolutionary selection was the individual organism. Stephen Jay Gould (2002), in consequence of Mayr (1940, 1942), considered the species as unit, but also widened the scope including genes, cell lineages, organisms, demes, species, and clades. However, forcefully Gould rejected evolution of what he called "large items in nature," in our essay implying HLS Earth as the most prominent "item." Reluctantly Gould admits that communities maintain some functional coherence and boundaries to be defended, as well as some potential of splitting off "daughter communities." However, he has fundamental reservations accepting such ecological units to be Darwinian agencies. Arthur George Tansley (1935) went a step further, when defining "ecosystem" as a natural unit. Neither he accepted, however, highly integrated ecological entities as akin to organisms. Highly integrated HLSs in the views of Gould and Tansley neither are evolutionary individuals nor units of selection, because the generic triple of mutation, heredity and selection did not appear to them as realized in such systems. Such reservations are the challenges we will face and discuss as we move on to HLS evolution in the following, including HLS Earth in Sect. 4.

In essence, evolution as a trial-and-error process maintaining system viability proceeds via adaptability capacities relative to environmental changes. As living systems act within their environment, the latter is constantly subject to change. In turn, they are directly or indirectly affected by other co-occurring systems as part of the same joint environment, mirroring principles such as niche construction, stigmergy, or social learning. These consistently hold for within and between-HLS conditions. Hence, each of the involved systems (or HLSs) perceives environmental changes. As already addressed, some of the changes might be predictable (e.g., seasonal or daily cycles), whereas others are random (e.g., late frosts, wildfires). Adaptability must encompass both kinds of changes. Being adapted to a specific environment commonly means abilities of exploiting environmental opportunities or withstanding adverse restrictions effectively and/or efficiently. Notwithstanding, the system must preserve its creative potential to develop new solutions in view of unforeseen challenges. Following Maturana and Varela (1987, p. 115), "*evolution occurs as a phenomenon of structural drift under ongoing phylogenic selection. In that phenomenon there is no progress or optimization of the use of the environment, but only conservation of adaptation and autopoiesis*" (i.e., self-preservation). "*It is a process in which organism and environment remain in a continuous structural coupling.*"

Optimization is an a posteriori impression by observers, suggested by the self-regulation capacities inherent to biological systems between matter and energy fluxes. Optimization processes never reach ultimate stages, however, given the ever-changing environmental conditions. Only under premises of constancy in environmental conditions, being a matter of time scales defined again by observers, optimization towards sustainability of resource use is pretended. Such definitions may convey understanding of niche creation and related evolutionary specializations.

Nevertheless, pretended time scale-restricted optimization does not suffice to maintain viability (Bateson 1972), given ongoing interferences by environmental dynamics, including appearance of other, newly evolving biotic agents and unpredictable abiotic impacts. Such impacts may randomly affect the "super-system" (e.g., ecosystem HLS) a subordinate system (e.g., holobiont HLS) is embedded in, or holobiont-HLS sub-systems (e.g., mutualistic or parasitic associates). Given the restricted, definition-dependent scope of optimization, HLSs are required to maintain adaptability as anchored in "mutation," i.e., random variation in generic ways (creativity), in particular, beyond gene-centric scopes. A way of dealing with unpredictability is the development of new "prototypical action" as "trials" in view of their environmental consequences. Success of such "trials" must ensure new informational gain to be identified and adopted, while historical experience is to be saved during the execution of "trials." In cases of favorable outcomes, respective HLS variants tend to persist, whereas deteriorating variants gradually fade out (zu Castell et al. 2019). Precondition is, however, that HLSs stay sufficiently robust during "trials," i.e., "prototypical executions." Notwithstanding, extents of random change are limited. Resilience determines the level of perturbation HLSs may tolerate without losing internal integrity. Therefore, adaptation speed is framed by degrees of adaptability, which HLSs are able to attain without jeopardizing essential immanent characteristics.

As adaptability is not unlimited, sustainability is at risk, if required adaptive action approaches limits. Limits may be widened at best through acclimation based on physiological plasticity within the frames of given adaptations. Evolution solves such challenges by abandoning deficient HLS expressions upon reaching intrinsic default time-outs, commonly perceived as death. Hence, sustaining HLS-level experiences (i.e., the "song" sensu Doolittle) requires extra-individual HLS-level storage or "memory" (i.e., independent of "singers"). Surely, some information is individually DNA-based, however, additional information transfer is required across time-outs of "singers" in preventing HLS-level time-out of the "song." Moreover, an additional source of creativity beyond DNA-associated mutations is needed to allow execution of new HLS "prototypes" and their adaptations to speed up. The success of HLS evolution critically depends, therefore, on alternating sequences of creative diversification and subsequent memorization with temporary consolidation, altogether creating new "trials" with chances of evolutionary success.

Regarding consolidation, trial-and-error processes are HLS-challenging. Trial phases are dynamic but rely on temporary system robustness, which enables plasticity in responsiveness (Souza and Lüttge 2015). That kind of premise is remarkable the more so, as any living system is prone to instability for thermodynamic reasons. Once more, capacities of self-organization and self-regulation are crucial, particularly holding for HLSs (zu Castell et al. 2019), based on informational exchange with the environment (Netter 1959). On such grounds, complex dynamic flow equilibria emerge, representing non-linear pseudo-steady states (Glansdorff and Prigogine 1971; Nicolis and Prigogine 1977; Lüttge 2016; Lüttge and Scarano 2019; zu Castell et al. 2019). Instabilities in self-organization are crucial in providing the ontological core for driving both developmental (Devo) and evolutionary processes (Evo; Schmidt 2015, 2019; Sect. 3.3). "*Stability and instability are not disjunct, but stability in complex* (living) *systems … has to be regarded as a derivative of instability*" (Schmidt 2019). Thus, phases of consolidation allow HLSs to temporarily maintain their overall structure and functionality before being "re-worked" during subsequent creativity phases of instability. Inheritance of adaptations links creativity and consolidation phases, warranting continuous transfer of information. As a result, we see the action of natural selection in retrospective as an autonomous process without being driven by any HLS-intrinsic or extrinsic force. Rather, selection results from HLS variability as the outcome from evolutionarily relevant processes.

The sequence of repeated HLS alterations between creativity and consolidation phases is mirrored by gradually advancing HLS revisions over time. Following the adaptive cycle (see Sect. 3.2), upon each HLS breakdown and reorganization follows a period of exploitation and accumulation of information. Recalling holobionts sensu stricto (as giving rise to the HLS concept and being HLSs themselves) with a central host within the associated species community, the host's ontogeny and lifespan acts as pulse generator for creativity-consolidation cycles. In such terms, the entire organismic community becomes the entity of co-evolutionarily relevant selection. Examples of holobionts sensu stricto may be as straight as two unicellular organisms where the ciliate *Paramecium bursaria* acts as host of endosymbiotic *Chlorella* (Karakashian 1975; Smith and Douglas 1987), lichens as symbiotic units of fungi and algae, biofilms of bacteria (Mathesius and Watt 2010) or many other quasi-organisms or quasi-species such as trees with their mycorrhizae and endophytes/parasites or even humans. The latter harbor several hundred different species of bacteria, most of them being preconditions and in support of human existence upon joint evolution, i.e., co-evolution. All such units as a whole, as referred to above, are subject to selection.

The extended definition of HLSs beyond holobionts sensu stricto regards any association of interacting agents in general as an evolutionarily relevant entity. Hence, any HLS, irrespective of ecological scale, represents a unit of selection as a whole, including the intrinsic interaction patterns and networks. Consequently, any short-term changes in interactions have the capacity of driving, via selection, long-term HLS differentiation, linking developmental (DEVO) with evolutionary processes (EVO) at HLS level. Based on repeated sequential selection, evolution is an essential and intrinsic aspect at HLS level, conceptually irrespective of the scales of holobionts sensu stricto, ecosystems or the biosphere as HLS Earth. Hence, evolutionarily relevant selection neither acts at the level of individuals sensu Darwin nor of species as suggested by Gould (2002).

3 Mechanisms of HLS Evolution

3.1 Co-evolution and Selection

How does the quest for units of selection relate to the phenomenon of co-evolution? The answer is straight regarding holobionts sensu stricto, in particular, at their subordinate scale. Here, the associated organisms tightly live together to extents which enable a new individual entity to emerge, namely, that of the holobiont. Such entities evolve as a whole, they are units of selection. In doing so, their subordinate agents, as a consequence of their interactions, pass through co-evolution. An evidently illustrative example of holobionts are lichens, symbiotically harboring two interacting subordinate species, i.e., one fungal and one algal agent, while developing specific adaptations as a whole. Lichen holobionts differ morphologically, biochemically and ecologically to extents that conventionally allow ranking as species on their own. The power of the concept of HLSs enfolds when considering higher scales or when subordinate agents are spatiotemporally more loosely integrated in terms of behavior and ecological functions. Flowers and their pollinators may serve as examples here. In such cases questions about holobiontic co-evolution become challenging.

Regarding holobionts sensu stricto, it is increasingly understood that individuals of plants or animals do not persist and evolve per se, but as central actors within communities of interacting other individuals from diverse associated species. Such communities represent one integral genome, termed hologenome (Zilber-Rosenberg and Rosenberg 2008), and jointly adapt to changing biotic and abiotic environments, i.e., the associated agents proceed through co-evolutions. Such co-evolutionary interactions include those with the abiotic environment. Through those interactions, the genome of each species changes, and so does the hologenome, implying that both loss of associates and acquisition of new ones adds to the hologenome-level and co-evolution. Hence, the holobiont actually is the evolutionary unit and subject to multi-level selection. It is important to note that as part of the interaction network also site-relevant abiotic information is forwarded (inherited) over time. Note that a genome obviously integrates experiences gained through interaction with a certain environment. Consequently, the genome can also be interpreted as a hypothesis on the environment to be eventually encountered. For example, the genome of *Desulfuromonas acetoxidans* encodes chemical pathways to reduce sulfur. Thus, its genome encodes the hypothesis of encountering sulfur in the environment. Holobionts ultimately embody, therefore, extended spatiotemporal dimensions, reaching beyond the lifespans of individuals (i.e., the “singers”). Their co-evolution speeds up the adaptability of the holobiont in support of the central agent.

Genomes are a powerful way of encoding information concerning biochemical mechanisms of interaction within a given environment. Thus, evidently also regarding HLSs at ecological scales higher than holobionts sensu stricto hologenomes are encountered. Such a view, of course, supports deriving the HLS concept from that of the holobiont, with the exception, however, that within HLSs all agents are equitable in interactions. Depending on their scale, such HLSs integrate subordinate holobionts in the case of ecosystems, or ecosystems in the biosphere (as representing HLS Earth). Consistently, any HLS possesses a hologenome as reflecting species co-evolution and changes in species composition. However, inheritable information is also stored beyond DNA-based genetics, and conveyed by HLS-level structural and functional alterations (including the geochemical environment), altogether preserving and forwarding outcomes from historic contingencies. Social learning provides an obvious example of perpetuating contingencies, e.g., as can be seen in the development of the theory of evolution (see Sect. 2.3). To some extent, informational storage beyond the HLS hologenome resembles principles of epigenetics at cell level. Alike any holobiont, HLSs are open dynamic systems and reflect capacities of self-organization and regulation in energy and matter exchanges with environments, sustained by feedback control within the interaction networks. Initially, positive feedback control at least marginally must outweigh negative feedback to sustain self-organization and regulation as pre-requisites for forwarding (inheriting) HLS-specific information on the interaction network. Nevertheless, information is modified by sequential selection under continual environmental impacts, fostering adaptability and shaping adaptations during HLS evolution.

On the basis of symbiotic and co-evolutionary community units, group selection is encountered as a key feature of HLS evolution. Hence, being co-evolutionary systems, any HLS as a whole is subject to selection. Notwithstanding, even after one decade of developing the holobiont theory, the dispute about what to rate as unit of selection has not been settled (Zilber-Rosenberg and Rosenberg 2008, see above Sect. 2.5). One objection is that specificities of close host-microorganism co-evolutions are merely upheaved relative to the commonly divergent selection of interacting species (Skillings 2016).

3.2 Adaptive Cycles

The adaptability noted here during HLS evolution follows non-linear dynamics. Evolving HLSs naturally pass through consecutive phases of growth and release, constituting a dynamic pattern generated by the adaptive cycle (Holling 1986). Adaptation builds on potential being generated via the activity of the HLS. Continuing survival reflects the systems capability to cope with various environmental changes. By continuously building on "successful" strategies, the system increases its internal level of organization, termed connectedness. Resilience as a third factor counterbalances an increased level of adaptation, and thus initiates the interplay of adaptability and consolidation. The better one HLS reflects and copes with the specificities of its environment, the more adaptation and ecological niching are advanced. Increasing progress in complying with the environment, conversely, enhances the vulnerability of HLSs to alterations. As the environment is intrinsically dynamic due to its multitude of biotic–abiotic interactions, any HLS becomes an "*accident waiting to happen*" as comprehensible by the adaptive cycle metaphor (Fig. 2a, Gunderson and Holling 2002). Based on interaction assessment in complex systems, the cycle is observable in biological as well as social systems (zu Castell and Schrenk 2020). Thus, the dynamic interplay between consolidation (as taking place during phases of breakdown and reorganization) and accumulation (as taking place during phases of exploitation and conservation) and resilience constitute a natural impulse generator of alternating phases of predictability (i.e., information accumulation) and stochasticity (leading to aggregation of information). The question is about the extent of information conservable within systems during transitions between release and reorganization (sensu heredity), i.e., breakdown (at least partial "death") and renewal (at least partial de novo "re-birth") of HLSs, respectively.

The adaptive cycle thus results in a mechanism of sequential selection. Advancing as systemic unities subject to sequential selection (of generic quality), HLS evolution is conveyed through adaptive cycling, even in the absence of resource-related competition, or if persistently restricted to same sites (e.g., for millions of years in cases of rainforest evolution). Selection results from the system being urged to maintain its internal stability upon constantly changing environmental conditions.

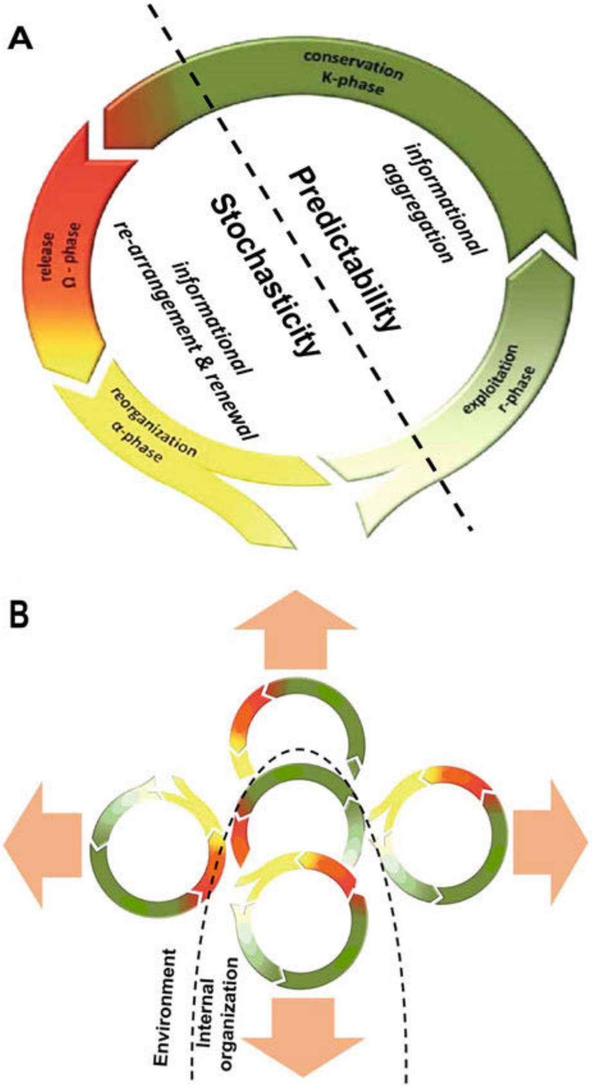

Fig. 2 (**a**) The adaptive cycle as natural pulse generator for alternating phases of creativity (stochasticity) and consolidation (predictability) as part of a trial-and-error process. Information is aggregated during the exploitation and conservation periods, whereas being re-arranged and renewed during periods of release and reorganization. The cycle transmits information via processes of generic heredity. Phases are resource exploitation (r), resource conservation and consolidation (K), resource release (Ω) and reorganization (α). The α – r transition is crucial for evolutionarily transforming concurrent HLSs and being transformed by such (split open ends of cycle), providing grounds of generic mutation. Each run through the cycle acquires progress in HLS adaptation and evolution. (**b**) Panarchy visualizing interaction between adaptive cycles, both of three co-occurring HLSs (horizontal arrows) and, in parallel, of the HLS at mid-position. Interaction proceeds across subordinate and superordinate cycles at adjacent ecological scales (downwards and upwards, respectively; vertical arrows). The resulting cross-scale interrelationships, being multiple in

Thus, we also need to broaden our understanding of the notion of competition (see Sect. 4.1, below). It is claimed that the initial stage in HLS development is characterized by intense resource exploitation, termed r-phase (see Fig. 2a). The terminology orients at a corresponding stage in population growth. The r-phase gradually develops into the K-phase, when HLSs attain a stage of resource conservation and consolidation (comparable to population dynamics of K-strategists and climax stages of natural successions). r- and K-phases profit from the HLS-level self-organization and regulation capacities. Eventually, signs of natural decline develop, characterized by resource release from structure and function, which indicates arrival at the Ω-phase. During that phase, the decline conveys re-mixing of resources (sensu "creativity"), being enabled through failure in sustaining high levels of order with their associated energetic demands. Previously acquired information gets lost to some extent, depending on the robustness of informational storage (memory), while new capacities arise for informational re-structuring.

The Ω-phase leads to HLS reorganization, termed α-phase, which is crucial to further HLS development. At the α-stage, the system has become altered, so that two options arise, depending on the extent of change. Either, the system is ready to commence the next run through the adaptive cycle, if changes mainly led to another modified HLS variant. Or, the changes created a thoroughly different HLS type. In such latter case, the novel type leaves the adaptive cycle and initiates a new one on its own (imagining, e.g., the release of a new ecosystem-scale HLS). In addition, HLS fragments may initiate new adaptive cycles on their own. In either case, the HLS variant before commencing the next turn may merge at the beginning of the r-phase with other HLS types or variants (Fig. 2a). It is, in particular, the α-phase and the transition between α- and r-phases where new information can enter the system. Increased stochasticity rather than stability provides grounds for (generic) mutation to change the system. HLSs can be modified by others, underlining overarching interactions as part of the HLS concept and initiation of selection. Mechanisms of generic selection become apparent between and within HLSs as promoting evolution, even if persisting on same sites (see above) and without merging with other cycles. This means, merging is not mandatory, as may result from cross-HLS competition, and hence, neither competition per se is coercive. Widening the perspective, also ecosystem-level HLSs evolve upon each run through adaptive cycles. In doing so, such HLSs draw and emerge from functions, performances, and adaptations, which had been acquired during previous rounds and/or interactions with other concurrent HLSs. Such latter interaction is not mandatory, however, and is irrelevant, in particular, in the case of HLS Earth. Likewise, any HLS can exclusively evolve through repeated cycling between reorganization and adaptation, intrinsically reflecting processes of sequential selection.

Fig. 2 (continued) spatiotemporal terms, altogether constitute the essence of panarchy. Note that co-acting adaptive cycles do not necessarily need to be temporally synchronized (according to Gunderson and Holling 2002 as adapted from zu Castell et al. 2016)

The mechanism of adaptive cycles is extended by the panarchy concept (Fig. 2b) according to Gunderson and Holling (2002). HLSs may interact "horizontally," while concurrently proceeding through their adaptive cycles each, without necessarily being synchronized in temporal progress. Crucial for selection and, hence, for evolution are the phase α- to r-transitions between co-occurring HLSs. The other dimension acts "vertically" within the interaction network, extending across adjacent scales of ecological organization (Fig. 2b). Focusing on a given HLS, the super-system it acts within as well as other HLSs acting horizontally constitutes the system's environment, whereas the sub-systems the HLS consists of define its internal organization. The integration of horizontal and vertical dimensions embodies the core of panarchy. Both dimensions drive HLS evolution, including the ecosystem scale, i.e., horizontally through interaction between adaptive cycles, and vertically during each turn within adaptive cycles. Overall, the panarchy concept of HLS evolution allows for deriving Darwinian principles as functionally resulting from principles of adaptation within changing environments.

In conclusion, adaptive cycles naturally subdivide the temporal development of HLSs into alternating phases of consolidation (i.e., r- and K-phase) and creativity (i.e., Ω- and α-phase). While the transition from r- to K-phase constitutes a period of exploitation by a newly instantiated HLS prototype, the breakdown during the Ω-phase represents partial "death," at least, of the prototype. That kind of functional disintegration, which releases resources and re-opens or newly offers ecological niches, is the pre-requisite, however, for a subsequent α-phase to yield novelty, i.e., new prototypes. Note that the adaptive cycle follows non-linear dynamics without conceptually requiring HLS evolution to linearly proceed in spatiotemporal terms.

The neo-Darwinian theory of evolution commonly perceives fitness of an organism in terms of the number of offspring. Such a view, however, falls short within the scope of HLSs. Any HLS may release progeny during adaptive cycling, as such offspring may proceed then in independent cycles even beyond the ultimate breakdown of parent HLSs. Such releases are just one way of preserving gained information, namely, through transition to reorganization in a new HLS generation. Notwithstanding, it is not the number of daughter HLSs per se, which defines fitness, but the unfolded capacities of initiating new prototypes, expressed by novel HLSs or newly created and covered ecological niches. In such terms, fitness also comprises vegetative proliferation as constituted by cloning.

Very large clones, for example, are found among trees of the genus *Populus*. In the Qira Oasis of the Taklimakan desert (Hotan Province, Chinese Xinjiang Uygur Autonomous Region, China) a clone of *Populus euphratica* Oliv. of a size of at least 4 ha has been identified (Bruelheide et al. 2004). The largest clones known in the world are of *Populus tremuloides* Michx., i.e., in the Wasatch Mountains in Utah, North America, with 42.9 ha (Grant 1993) or even larger elsewhere (81 ha: Kemperman and Barnes 1976). When we consider genetically identical members of such clones as individuals, such addressed clones are evidently HLSs. They successfully cope with – and niche in – newly encountered site conditions showing their fitness without any sexual progeny. Thus, offspring is not mandatory for HLS fitness and evolution to proceed as affirmed by HLS Earth. What is important, however, is a way of getting rid of some contingency and a chance for some change to enter.

In any case the identification of some concrete HLS prototype (inevitably being a choice made by the observer) is inherently bound to naturally occurring sequences of consolidation and creativity within or – interrelated – across adaptive cycles. Seeing such cycles acting as pulse generators, evolution is perceivable as being "at work," perpetually rebuilding modified prototype expressions until replacement under the dynamic ecological settings. Let us recall the key point that selection emerges from the perpetual interaction of all involved agents, including the abiotic environment, so that in such sense, natural selection is an outcome from self-organization. Whether considering such outcomes as "new HLS generations" or even "new types of HLS" is a matter again of the observer's perspective. The kind of circulating rebuilding and renewal is the core of selection and, hence, evolution as sequential processes, holding for any HLS.

Theoretical modeling reveals two types of destabilizations with significant changes in interaction networks of evolving HLSs. The disruptive form of a "quake" arises from a labile agent leading to the replacement of the stabilizing network core (cf. Arthur et al. 2017). Instead of replacement through a harsh "quake," the destabilizing agent may link and eventually merge with the core to an extent that environmental capacities prevent ultimate breakdown of the core's network integrity. Simulating adaptive cycles on the basis of abundance data permits estimating such "take-overs" as reflected by the degree of vibrancy in cycle phases (zu Castell and Schrenk 2020). It seems that moderate vibrancy fosters HLS plasticity and, hence, capacities of resilience from environmental impact.

Considering such "take-overs," it becomes apparent that HLS evolution does not necessarily progress gradually, in particular, if accompanied by harsh "quakes." The idea of punctuated equilibria complementing the view of phyletic gradualism was formulated by Eldredge and Gould (1985; cf. Gould 2002). Punctuated equilibria result from co-evolutionary avalanches (Bak and Boettcher 1997). Such bursts of adaptive changes characterize self-organized criticality, a phenomenon being well studied in the context of non-linear dynamics (Bak 1999). The Cambrian explosion of species initiation exemplifies such an avalanche at the biospheric scale similar to phenomena associated with mass extinctions, which repeatedly have opened opportunities for often newly arising taxa in gaining ecological dominance. At genome level, speciation may result from similar events, as according to Gould (1980) speciation may typically result from rapid genome reorganization. Endosymbiosis provides an overwhelming example, showing interactions between single organisms to eventually lead to a dramatic change at high ecological scales, eventually in the biosphere–atmosphere interaction of HLS Earth (e.g., Margulis 1998). In that latter case, similarity exists to "Gaia" (Lovelock 1979; Matyssek and Lüttge 2013; Doolittle 2017; zu Castell et al. 2019).

"Gaia" was posed on natural-scientific grounds as a hypothesis that states planet Earth to be "*a complex entity involving* …. *biosphere, atmosphere, oceans and soil*" and "*self-regulating with the capacity to keep our planet healthy by controlling the chemical and physical environment*" (Lovelock 1979). "Healthy" meant biospheric self-regulation to sustain the planet's capacity for harboring life. In particular, it is claimed that life, while altering the abiotic environment, also gains advantages from the exerted alterations for coping with stress. Being dynamic and stabilized via feedback coupling with the geochemical world, the biosphere is to transform the entire planet towards one self-containing ecosystem, i.e., "ecosystem Earth." That view acknowledges "ecosystem Earth" as a self-regulating interaction and, hence, communication network between functional and structural agents. Below, we will show that the premises of HLSs are fulfilled and, therefore, the view of HLS Earth. Being conceptually anchored in the holobiont definition, HLS Earth may be claimed even as the ultimate case of one being-like entity (cf. Sect. 4.2).

The HLS Earth perspective conceptually exceeds the Gaia hypothesis through solid grounding on ecosystem-level cause-effect relationships and, hence, ecological mechanisms. These represent the informational essence of the intrinsic process-based, multi-level interaction network and its variation over time. Consequently, global HLS variants emerge, which mirror HLS evolution as based on adaptive cycling since the planetary accretion phase, with global-scale generic extensions of mutation, heredity, selection, and adaptability. In such way, HLS Earth evolves as continually complying with, i.e., adapting to, shifts within the biogeochemical interaction network. The (co-) evolution of the global interaction network between the biosphere and the geochemical "spheres" (i.e., the physicochemical compartments of atmo-, hydro- and lithosphere) is particularly comprehended by evolutionary developmental theory (EvoDevo) and extended in ecological terms by the EvoDevoEco concept.

3.3 The EvoDevoEco Concept

From the preceding paragraph two perspectives on interaction emerge, i.e., ecological interaction and developmental interaction. Considering evolution, do these constitute differing aspects, or can we reconcile them towards understanding the evolution of HLS Earth? Evidently, HLS Earth is under continued *development* (Devo) as facing the perpetual changes in biogeochemistry. Temporary stages in development and biogeochemical interactions may be viewed as consecutive "phenotypes" or, generically, "infotypes" sensu "prototypes" of HLS Earth, as detailed before. The interactions are biased by previous contingencies during Earth-historical geochemical eons and sporadic biological extinctions (Matyssek and Lüttge 2013), the after-effects of which are traded over time through generic qualities of biogeochemical memory and heredity. Environment links to *ecology* (Eco), and HLS Earth as a whole becomes a unit of *evolution* (Evo; cf. Sect. 3.4). The field of evolutionary developmental biology (EvoDevo) integrates research on developmental biology and evolution, although basically conceived for the scale of individual organisms (Gilbert 2013; Minelli 2009). Due to the selective pressures of the environment as the driving force of evolution, ecology becomes an inherent component already at the said scale. Although our view on evolution focusing on agents interacting with their environment naturally encompasses ecological interaction, we stress the importance of including the ecological paradigm by extending the concept towards EvoDevoEco (Müller 2007; Gilbert and Epel 2009). This extension becomes increasingly relevant towards the high ecological scales (Lüttge et al. 2012), while covering different time vectors.

As evolution constitutes a sequential process, i.e., based on sequential selection under the dynamics of consolidation and creativity phases, several time vectors are distinguishable. Such vectors may vary in duration, as represented in ecological terms by (sensu stricto physiologically) reversible *acclimation* and irreversible long-term *adaptation* at short- and long-term scales, respectively (also see Sect. 3.4). Adaptation is encoded as system-level informational storage, which may be, but not exclusively, DNA-based. Beyond DNA levels, also additional adaptive alterations in structure and function pass along time vectors, i.e., are inheritable, and are of informational system-level relevance. In the last decade, conservation of environmental information in terms of epigenetic memory (see Thellier and Lüttge 2013; D'Urso and Brickner 2014) has gained attention, being one basic mechanism in informational storage and transfer at HLS level. Adopting the HLS perspective, all kinds of informational storage constitute, therefore, system memory. This also holds for *acclimation*, when viewed beyond physiological scopes as reversible alterations within frames of irreversible *adaptation*, given that both phenomena are defined in generic terms.

Irreversible long-term adaptation is fixed, e.g., in the genome, whereas reversible short-term acclimation operates predominantly within the frame of physiological plasticity and, hence, the scope of gene expression. The prolonged time vectors of system-level adaptation convey all kinds of encodable informational modifications (i.e., both beyond and within DNA scales) and, hence, denote evolutionary progress (Evo). Short vectors denote acclimation and reflect development (Devo). Notwithstanding, both adaptation and acclimation are driven by environmental, ecologically relevant dynamics (Eco).

Epigenetic regulation (see below Sect. 3.4) can construct a link between adaptation and acclimation, both able to occur directly in response to environmental cues and to be inherited. That kind of regulation leaves DNA unchanged, but acquires information by modulating nucleosomal histones and chromatin by acetylation and methylation, as the latter silences genetic information (Chinnusami and Zhu 2009; Yaish et al. 2011). Epigenetic mechanisms enable developmental responses to environmental impacts, as incited by external triggers (Chinnusami and Zhu 2009; Chen et al. 2010; Daxinger and Whitelaw 2010; Verhoeven et al. 2010; Yaish et al. 2011). The triggered variations are inheritable and transferable throughout several generations (Bond and Finnegan 2007; Verhoeven et al. 2010). Such kind of modulated information, ultimately anchored in unchanged DNA, may persist over variable periods of time and constitutes a molecular, namely, epigenetic memory (Bond and Finnegan 2007, for review see Thellier and Lüttge 2013). EvoDevo and EvoDevoEco overrule the strict neo-Darwinian correspondence of phenotype and genotype, since they spread out a continuum of changes in interaction patterns, resulting in varying degrees of temporal stability.

Two examples from plant biology may illustrate the essence of differently extending time vectors regarding EvoDevoEco relationships.

1. In dense tropical rainforests, adult individuals of tree species are adapted to and require the high solar irradiance in the canopy for persistence (Evo). Seeds upon arriving at the forest floor rapidly germinate and develop seedlings (Devo). The seedling stage is typically short in absolute terms of time. However, in the darkness of tropical forest floors, seedlings may persist up to several years, before accidental canopy gaps allow development with rapid growth towards tall tree sizes (Devo). Only then the sun-exposed upper canopy is approached with conditions, which adult stages are enabled to cope with through evolution (Evo). For example, the canopy species *Richeria grandis* Vahl. has a germination time of 20 days and cotyledon lifespan of 2.5 years, with primary leaves lasting over 3 years (Flores 1992; Lüttge 2008).
2. A prominent example of adaptive ecophysiological plasticity is the Crassulacean Acid Metabolism (CAM), regarding photosynthetic adaptation to limited water availability. Most plants (except for CAM plants) take up carbon dioxide (CO_2) into leaves via controllable stomatal pores during daytime when solar energy is abundant. However, daytime opening of stomata is inevitably accompanied by simultaneous loss of water vapor, bearing desiccation risk under high irradiance and temperature. CAM plants have evolved, as a result of adaptation, a metabolic cycle, which allows stomata to stay closed during daytime, while opening stomata and incorporating CO_2 at night when desiccation risk is minor. Carbon is stored at night first as organic acids, which release CO_2 only during the subsequent daytime behind closed stomata, when notwithstanding light is used for assimilation. The CAM process constitutes adaptation, although being less productive than instantaneous CO_2 uptake and assimilation by the prevalently regular photosynthesis. Some plants can choose between CAM and regular photosynthesis. This depends on their acclimation capacities to variable water availability (Lüttge 2007). Such switching with activation of acclimation on the background of adaptation implies developmental plasticity.

HLS Earth development is closely related to the development of its environment, as the latter is part actually of the HLS concept. In such terms, fitness for the survival of HLS Earth, sensu the claimed comprehensive long-term integrity across biotic and abiotic agents, is an outcome of evolution. Such conclusion is consistent with the EvoDevoEco concept, stressing the role of development under biotic and abiotic impacts and, hence, corroborating uniquely singular systems like HLS Earth to be subject to evolution. In fact, Charles Darwin already underlined the role of developmental modifications during the process of natural selection and establishment of fitness. He considered development and embryology as the most important aspects of natural history (Friedman and Diggle 2011; Matyssek and Lüttge 2013). The "biogenetic law" according to Ernst Haeckel with his understanding that ontogeny recapitulates phylogeny links the ecologically relevant short- and long-term time vectors as expressions of adaptability and, hence, evolution. Or in short, Evo and

DEVO are intimately interwoven with each other and with ECO (Müller 2007; Gilbert and Epel 2009; Lüttge et al. 2012).

3.4 Perpetual Adaptation and Evolutionary Progress of HLSs

Evolution theory as explored by the modern synthesis (Dobzhansky 1937; Mayr 1959) or theoretical ecology (MacArthur and Wilson 1967; Odum 1971) suffers from inherent limitations that arise from focusing on one single concept, namely, that genes encode all forms of life and related actions. As population genetics adequately explains Mendelian patterns of inheritance, reducing evolution to the gene as sole informational carrier diverts the focus from phenotypic interactions (see also Noble 2015; Torday 2015). Other shortcomings prevail on the ecological side. Classical ecological theory with its core concepts of ecological succession and niche theory lost track of the historical contingency, which is crucial in shaping ecosystems. Popper's posed alternative of active Darwinism (Popper 1986, 2013) aims at restoring the central role of the phenotype as being subject to biological activity and, hence, ecological interaction as the stage of selection. For advancing the comprehension of units of selection and evolutionary progress at HLS level, the calls for differentiating evolution theory must be recognized (Kimura 1983, Scherer 2017; see above Sects. 2.3, 2.4, and 2.5). One central aspect to be acknowledged is, when viewed in retrospective, the prolonged course of historical contingencies as action grounds of sequential selection, being crucial for HLS evolution, particularly, at high ecological scales, including HLS Earth.

Generic heredity comprises all mechanisms of information transfer bridging the gap between accumulation and aggregation of information. While the Modern Synthesis accepts genetically stored information as sole mode of transgenerational memory, several non-genetic inheritance systems are known in the meantime (see, e.g., Danchin et al. 2011, Mesoudi et al. 2013). These can be roughly grouped into epigenetics and phenotypic plasticity, and ecological (and cultural) inheritance. Typically, none of them acts in isolation, but together within an interwoven network. There is a gradient, however, as to whether adaptations eventually foster evolutionary change. Pre-adaptations show that certain alterations might facilitate longer lasting evolutionary change. Similarly, the phenomenon of convergence demonstrates that environmental conditions are inducing development of certain biological solutions.

One illustrative example are pre-adaptations, being of minor evolutionary significance under the environmental scenario of their origination, but boosting adaptability and compliance with alterations during subsequent evolution. Instructive in the case of HLS Earth is the appearance of bi-polar filaments during the evolution of aqueous green algae, leading towards heterotrich forms in the order Chaetophorales and classes of Coleochaetophyceae and Charophyceae. Genera of the Chaetophorales, such as *Stigeoclonium* and *Fritschiella*, evolved both upright and, at the ground, branched filaments. The resulting growth habit marks the path to the bi-polar gestalt of stationary vascular plants, foreshadowing the functional differentiation between roots and shoots of Higher terrestrial Plants. In retrospective, evolutionary directedness is suggested (Layer and Lüttge 2020; Lüttge 2020), based on the pre-adaptation of algal filaments that enabled the subsequent evolution of Higher Plants, when forced to further adapt to the newly faced aerial conditions. That kind of evolutionary sequence ultimately triggered the conquest of land and boost in diversifying terrestrial life, becoming a cornerstone, at the same time, in the evolution of HLS Earth as a whole.

Phenomena as outlined above are inherent to evolving convergences. Biology is full of examples, where selection pressure by similar environmental conditions led to the independent evolution of similar structures and functions in phylogenetically remote lineages of life. In the plant kingdom, one extreme example is the evolution of consistent strands of sieve elements and sieve tubes in marine giant kelps of brown algae (e.g., genus *Macrocystis*) and terrestrial Higher Plants, respectively, in both cases driven by the need for long-distance transport of assimilates through plant bodies. Another prime example is convergent stem succulence, the evolution of which occurred independently in several phylogenetically distant plant families, including Cactaceae and Euphorbiaceae. It is hard to distinguish their succulent stems, if it were not for flower morphology and the presence of latex in Euphorbiaceae, but absence in Cactaceae. Their convergence even does not only exist in phylogenetic, but also in geographic terms between remote continents, envisioning vicariating Euphorbiaceae in the old world, but Cactaceae in the Americas. A prominent example amongst animals is the kind of eye with lens and retina that has evolved to almost identical anatomies in worms (Alcipodia), mollusks and mammals, including Man (Morris 2003, 2008). The evolution of the eye exemplifies instructively, how evolution has formed various eye systems developing from an interplay of the *Pax6* gene family within different environmental scenarios and through a complex interplay of molecular interactions (see, e.g., Zuker 1994, Fernald 2004). Even cognitive abilities related to culture show convergence. Such examples affirm that under similar selection pressure apparently one ideal set of adaptive solutions exists, regardless of evolutionary kinship or global region. Numerous examples suggest convergence to be the evolutionary rule rather than exception (Morris 2003, 2008). Convergence illustrates retrospective directedness towards closely corresponding adaptations, even though resulting from separate contingency courses as the grounds of sequential selection. That impression even led to the choice of the provocative title “Jenseits des Zufalls” in the German translation of Morris’ (2008) book (*Beyond Chance*). In conclusion, evolutionary progress inextricably draws from sequential and irreversible historical contingencies, irrespective of ecological scales and their kinds of HLSs, hindering fall-back to previous states and annihilation of advancement. Such principle must be kept in mind also in view of evolution at ecologically high HLS scales, as constituted by ecosystems or the entire biosphere.

Adaptive cycles are a basic phenomenon in HLS evolution at any scale of ecological organization (see Sect. 3.2). To evolve, it is not sufficient to run through consecutive cycles of organization and release. Evolution proceeds by amplifying initial random variation. Initially, a difference lies in subtle variation, allowing one system to persist more successfully than another. Via transmission of information throughout the Ω- and α-phases (see Sect. 3.2), such a subtle bit of information remains in the cycle and can subsequentially be amplified. As information must be kept and accumulates over time in order to generate progression, HLSs rely on mechanisms to store and transmit information throughout several loops of adaptive cycling. Particular focus needs to be directed to the transfer process between consolidation phases (i.e., from one identifiable HLS "prototype" to the next). Hereby, the Ω- and α-phases provide particular challenges with their high unpredictability during breakdown and resource release, as information, even though being modified, needs to be maintained during the transitional creativity stage towards new resource use and HLS re-arrangement.

One way of informational persistence is to bypass perturbance, i.e., robust memory is to be created. Information encoded in DNA is the most prominent form of memory, being used ubiquitously in all living organisms. Overall, informational memory, irrespective of the way of realization, is a basic property of all life (Progam, see Sect. 2.2). In molecular and metabolic terms, memory is created through substrates and their transports, as metabolic inductions stay stable, i.e., are memorized for some time, even if triggers intermittently disappear. Such principles already exist in bacteria. In highly evolved organisms including plants types of memory are manifold (Thellier and Lüttge 2013; Thellier 2015, 2017a, b). Pathways exist with informational storage through habituation, familiarization, or sensitization, often called priming and summarized as learning. They can be followed immediately upon directly stimulated plant responses. Different are memory types in the form of complex networks with high degrees of informational integration and feedback, the more so, as storage and recall functions operate separately.

Cubas et al. (1999) demonstrated that a known variant in *Linaria vulgaris* Mill. breaking the typical flower symmetry can be traced back to epigenetic modification rather than a defined genetic lineage. Over time, various modes of epigenetic control have been unraveled, providing a rich toolbox for non-genetic, transgenerational inheritance (D'Urso and Brickner 2014). Epigenetic inheritance can occur in the germline, directly altering gene expression in progeny. In an indirect way, epigenetic modifications can also alter parental behavior such that the same epigenetic state is also induced in the offspring as, e.g., in postnatal maternal pup licking in rodents (Weaver et al. 2004).

Epigenetic mechanisms might further contribute to the ability of cells to deal with sudden changes of environmental conditions. DNA methylation in *Arabidopsis thaliana* (L.) Heynh. can generate substantial transgenerational variation (Schmitz et al. 2011). Variation is just one form of phenotypic plasticity allowing the organisms to cope with unanticipated environmental changes. Therefore, phenotypic differentiation contributes to evolutionary progression under natural selection bridging the gap to slower, genetic adaptation. Summarizing, the concept of a stable genome being transferred to the next generation is progressively being replaced by the idea of a body of information changing in a plasticly responsive way to environmental changes (Bonduriansky 2012).

Going further, Parsons et al. (2020) argue that phenotypic plasticity must be taken as a property of the developmental system as a whole. Indeed, the 3D genome provides a major driver of embryonic development (Hug and Vaquerizas 2018). Plasticity hereby provides a way for rapid physiological response, while genetic adaptability relies on randomly generated rare genetic events. The close interrelation between evolution and developmental processes has led to the formation of evolutionary developmental biology (EvoDevo, Sect. 3.3). One of the primary results of this young field is the elucidation of the homeobox gene clusters controlling cell partitioning in early embryogenesis as leading to compartmentalization in eukaryotes (Mallo 2018). In animals *Hox* genes are highly conserved master regulators leading to the plethora of organismal shapes and sizes. The homeobox provides a fascinating example of homology demonstrating developmental plasticity.

Developmental processes rely on two sources of parental heritable information. The parental genomes are embedded into two fully developed gametes, both upon mergence eventually providing a functioning cell metabolism. Both contribute to the developing phenotype. For example, inoculating a goldfish egg with a carp nucleus, the resulting fish exhibit characteristics of both phenotypes (Sun and Zhu 2014). Developmental plasticity can serve to anticipate environmental conditions, priming progeny to varying development. For example, Galloway and Etterson (2007) show, exemplifying understory plants, that offspring life history depends on maternal light environment, either shaded or sun-exposed in canopy gaps.

Reaching beyond single-species heredity, host-microbiome associations provide an example of generic heredity via species composition. Transmission of gut microbiota has been studied in many host organisms (see, e.g., Turner et al. 2013, Knoop et al. 2018, Moeller et al. 2018, Liu et al. 2019). Hereby, microbial associates play a major role in enlarging the functional repertoire of the host, priming the immune system, or governing physiological development (Hassani and Hosseinipour 2018). Microbes may also translocate through various parts of the organism as, e.g., from flowers to seeds (Mitter et al. 2017). Thus, environmentally acquired microorganisms can be passed on to successive generations, leaving an impact on ecosystem functions (Abdelfattah et al. 2021). For example, microbes play an essential role in plant growth and health (Liu et al. 2019). In particular, beneficial microbes have been shown to help their hosts coping with stress. Relying on pollinators for reproduction, the host-microbiome system also interferes with the plant-pollinator system (Engel et al. 2016; Rering et al. 2018). Thus, variations in the composition of associated microorganisms can reach up to the level of ecosystem functioning.

Species composition, in particular biodiversity, is fundamental to ecosystem functioning (Loreau and de Mazancourt 2013) and higher scales, providing one means of HLS memory. Adaptations to environmental changes as mirrored by altered diversity patterns represent HLS evolution, also recognizable in latitudinal species diversity gradients, and interpretable as the Dobshansky-MacArthur-Phenomenon (Brown 1995). Accordingly, biotic interactions are most limiting in tropical zones with highest biodiversity, whereas abiotic impacts such as by exacerbating temperature and aridity conditions become restrictive towards higher latitudes. Such macro-ecological trends are interpretable as informational coding on biotic/abiotic interaction patterns of ecosystem-HLSs towards HLS Earth. That way of storing information on HLS characteristics is consistent with physical laws describing abiotic self-organization as relevant during, e.g., wave formation or thunderstorms. Similarly, allometric scaling rules encode relations on energy use versus body size (Enquist et al. 1998; Enquist and Niklas 2002).

Another quality of ecosystem-HLS heredity is reflected by ecological niche development over time (Laland et al. 2017). Through modifying their local environment, species actively or passively alter the grounds of natural selection. The biodiversity-related niches differentiation theory (Gatti 2011, see Sect. 2.3) formulates a direct relation between the number of niches in ecosystem-HLSs and the extent of internal adaptability. Therefore, the variety of ecological niches carries HLS-level information on trophic networks, ecological functioning and species interaction along the time vector of sequential selection and evolution. Laland and Sterelny (2006) provide arguments for niche development as an evolutionary process on its own. Informational storage and heredity are consistent in their various mechanistic differentiations across HLSs up to the scale of HLS Earth, i.e., along the hierarchy of ecological organization. It is conclusive that interacting networks along the hierarchy of ecological scales end up at HLS Earth as the uppermost integral, continually evolving as an emerging, vertically bridged entity (Lüttge 2012, 2021).

A very special form of niche development is cultural inheritance by mankind, transmitted via social learning and imitation. Growing evidence suggests the cultural impact to affect the development of the genome of *Homo sapiens* (Laland et al. 2010). As an example, high body mass marriages raise predisposition of offspring to obesity (Ajslev et al. 2012). Social learning as a mechanism of cultural inheritance is widely found also in the animal kingdom. Passing on experience obviously constitutes a fitness enhancing trait (Giurfa 2012; van der Post et al. 2016; Reader 2016), again affirming generic qualities of inheritance. Within the scope of cultural evolution at the global scale, mankind may be considered as a particular kind of evolving HLS (Matyssek and Lüttge 2013), merging into the biogeochemical scope of HLSs. Ultimately, "HLS mankind" with all its biological and cultural features is an evolutionary outcome from HLS Earth, as it becomes an intriguing question today, to which extent this outcome may bias future HLS Earth evolution.

4 HLS Evolution at High Ecological Scales

4.1 Evolution and Competition Within and Between HLSs

In view of the outlined process of evolution, let us briefly revisit the aspect of competition in evolutionary development. Examples of HLS at community level evidently are ecosystems and biomes. Competition is traditionally understood as the challenge organisms are faced with in struggling for space and other limited resources. The caveat of such an understanding of competition lies in the necessity to assume somewhat stable ecological conditions. However, changing environmental conditions also continuously alter the struggle for resources. Thus, a more general idea of competition is required. Based on our understanding of evolution as being driven by the urge to sustain adaptability, *competition* can be understood as the challenge HLSs are faced with to maintain their internal stability despite randomly changing environmental conditions. This might be struggle of space and other resources, but can likewise result from the pressure to adapt to changing environmental conditions, i.e., compete sensu keeping pace adaptively with the progress of change. In this sense, it is readily conceivable that large-scale HLS indeed compete and are thus subject to selection.

Exemplified is the Amazonian core region, where rainforest never has naturally been replaced by other ecosystems during the past 55 million years. Has the Amazonian rainforest HLS actually been subject then to selection and, hence, evolution? Basically, ecosystem HLSs can be changed by abiotic impacts and/or ruled out by neighboring ones, which expand and intrude or newly establish within the original HLS. Such dynamics proceed through adaptive cycles, as already detailed above. Indeed, one such example is Amazonia. Cycles occurred here of the inversely expanding and retreating rainforest and savanna (Cerrado) HLSs during the past 55 million years, depending on climatic periods of contrasting humidity. At sites overrun by such advance and retreat cycles, HLS modification towards potentially ultimate replacement can be viewed as a reduced competitiveness in the sense of maintaining viability. Overall, the Amazonian rainforest HLS was not replaced, but evolutionarily modified, in particular, in its peripheral extensions. Changing the perspective, one can also state that during expansion/retreat cycles at affected sites the two contrasting HLSs evolved by being converted into an, at times, altered other HLS type. Under either perspective, the outcome today has resulted from evolutionarily relevant, competition-related internal and external interaction processes. The claim, therefore, is not devious that ecosystem-scale HLSs evolve through generic qualities of selection.

However, can one generalize evolutionary progress of rainforest HLSs worldwide, irrespective of regions? The ecological niches within rainforest HLSs justify to do so. Niches have not only been sharpened in their ecological profiles, but also their spectrum has increased via diversification, since the early days of rainforest expansions during the Carboniferous era up until today. Reflected is internal niches' co-evolution as continuously progressing within the rainforest HLS-level biocenosis. Another supportive argument is the ecological history of current rainforests, which can be traced back even over the past 150 million years in the case of the Malaysian tropics. Such high ages mean that current tropical rainforests are the oldest terrestrial ecosystems today on Earth, given their persistence on same sites, while sustaining niches' co-evolutions. Obviously, talking about competition in the traditional sense is not compelling in this case, whereas competition as facing the challenge of long-standing sustainability does make perfectly sense.

Given that the type of tropical rainforest HLS initiated its evolution at about 300 million years ago (i.e., in the tropics of the Carboniferous era), since then, that kind of HLS appeared to migrate geographically until arriving at current tropical locations. More realistic is the view that continental drifts trespassed underneath the tropical rainforest HLSs until these attained their current geographic positions. During that course and irrespective of view, the rainforest HLS permanently exerted and received selective pressure on and from the biotic-abiotic, i.e., biogeochemical environment, respectively. Also in this example, competitive phenomena in the sense of struggling for limited resources were not mandatory during that migration, however, with other HLS types being encountered, it is plausible that such were "absorbed" or replaced through processes of adaptation as conveying evolution via sequential selection. Mechanisms were consistent, then, with such known from the rainforest-Cerrado fluctuations in Amazonia. Notwithstanding, adaptive cycles of tropical rainforest HLSs have released "HLS offsprings" of other rainforest types during the past 300 million years, as exemplified by the temperate rainforest along the Pacific coast of NW-America. Such perception is plausible, as woody species communities migrated from the tropics during Earth history into other climate zones (Axelrod 1966; Brentnall et al. 2005; Kikuzawa and Lechowicz 2011).

Many of the ecological niches prevalent today were present already during the early beginnings, as low-latitude rainforest HLSs have always been determined by tropical conditions. Notwithstanding, species occupying ecological niches have changed since the Carboniferous era. The niche, e.g., of primary producers was originally dominated by diverse fern tree species, tree-like seed ferns, *Ginkgo*-type trees, and predecessors of modern conifers (*Cordaites*) instead of the currently predominant seed fern-derived angiosperm trees. In such terms, Doolittle and Booth's introductory maxim becomes understandable again that it is the song, but not the singer, which is evolutionarily important.

In parallel, both number and trophic spectrum of ecological niches have increased substantially, owing to rainforest HLS-internal species co-evolution, namely, mutual adaptability and resulting species augmentation. Competition within HLS even arises at the species level, because the other face of the medal of HLS-internal co-evolution is HLS-internal facilitation unless competition. Roux (1881) has noted that within individual organisms their organs indeed compete for resource allocation. In doing so, the organs facilitate each other in HLS manner. In analogy, niche development and selection are to be seen at ecosystem-HLS level.

Such progress led to the enormously high biodiversity with complex biotic–abiotic interaction networks, which are characteristic of tropical rainforest HLSs today and affirm ecosystem-scale HLS evolution as a whole. That outcome was even achieved, although several globally dramatic species mass extinctions during the past 300 million years (inflicted by terrestrial and cosmic impacts) have temporarily suspended the evolutionary advancement (Lenton and Watson 2011). Species substitutions and, nonetheless, continued species and niche diversification, however, perpetually intensified the HLS-characteristic interaction network, evidently not only mirroring ecosystem-scale HLS evolution, but also boosting it past mass extinctions. It seems that catastrophic mass extinctions were accelerators rather than terminators of life, promoting adaptive cycling and evolution of HLS Earth as a whole.

4.2 Global-Scale HLS Evolution Beyond Resource-Related Competition

At the highest level of HLS Earth, we have to consider competition in the sense as being given above. Indeed, the interaction system of atmosphere, lithosphere, hydrosphere and biosphere, forming the biogeochemical cycles that define HLS Earth, has adapted both to the gradually changing geochemical environment (including the ageing processes of the solar system and the Sun; Ward and Brownlee 2004a) and the increasing richness of the abiotic-biotic relationships. Atmosphere, lithosphere, hydrosphere, and biosphere demonstrate *compartmentalization*, as being part of the PICERAS criteria (see Sect. 2.2). Obviously, stabilization of the interaction system generated by the global main compartments relies on the energy provided by the Sun (*energy*, Sect. 2.2). An open question is, as to whether other planets or moons in and outside the solar system may have developed life and, hence, HLSs (Ward and Brownlee 2004b). Notwithstanding, such other planetary objects appear to be isolated through astronomic distances too large to compete with each other or HLS Earth.

Regarding HLS Earth's compartmentalization, the hydrospheric portion relevant within the biosphere outranges the corresponding lithospheric one in terms of claimed global surface area. Only 29% of total Earth's surface is terrestrial, whereas life rules the oceanic remainder from the euphotic zone downwards to deep-sea habitats. The oceans represent one coherent globe-spanning ecosystem, which may be structured into vertically arranged and/or zonobiomic compartments, as depending on research perspectives. Marine alike terrestrial biota form holobionts, with coral life forms, perhaps, as most prominent examples, giving rise even to timely views of holobiontic life as referred to at the beginning of this essay (Rosenberg et al. 2007; Zilber-Rosenberg and Rosenberg 2008). Actually, holobionts originated in aquatic environments as microbial biofilms. As encountered on land, holobionts are special cases of HLSs also in aquatic habitats. Hence, marine kelp forests or envisioned oceanic compartments can be regarded as HLSs at hierarchically high-ranking scales of ecological organization. In such terms, analogies with terrestrial HLSs are obvious. The global integral of marine HLSs yields the oceanic constituent intrinsic of HLS Earth. Why, then, the focus of this essay is directed to terrestrial HLSs?

One reason is the wealth of known and investigated terrestrial holobionts and the diversity of ecosystem-level HLSs on land. Another reason is that holobiontic life forms were the pre-requisite for life to conquer land under still life-hostile conditions (Layer and Lüttge 2020). That latter reason acknowledges the aquatic beginnings of HLSs with properties viewable as pre-adaptations to water-land ecotones. Microbial HLSs in the form of biofilms or with crust-building capacities were the first to explore such ecotones and incite advancements towards permanent terrestrial lifestyle (Matyssek and Lüttge 2013). Early advanced HLSs on land were, e.g., lichens, even occupying ecologically extreme niches, or mycorrhizospheres of Higher Plants. In both cases, HLSs rely on fungal associates of phylogenetically aquatic origin.

Without mycorrhizospheric HLSs, facilitating water and nutrient acquisition on land in addition to pathogen defense, Higher Plants would hardly have established in ways as familiar to us today (Layer and Lüttge 2020). Basically, HLS features are generic, irrespective of being embedded in aquatic or terrestrial environments. Focusing, notwithstanding, on terrestrial HLSs is owed to their evolutionarily attained complexity and diversity in adapting to the ecologically harsher conditions on land than in water. Hence, striving for genericness in understanding HLS principles is oriented at the challenging terrestrial settings, in particular, in view of the hierarchically high ecological scales with HLS Earth as the overall biospheric integral.

Given that outlined premise, doubts about HLS Earth as a system undergoing evolution are rebuttable, because evolution proceeds as long as HLSs succeed in sustaining themselves. This means, as they stay able to cope with prevalent biogeochemical settings and alterations, irrespective of resource-related competitive influences. Failure in compliance would lead to HLS demise, which has obviously not substantiated in the case of HLS Earth. Rather, generic selection pressure has forced towards compliance, progressively strengthening HLS adaptability to biogeochemical alterations. Such kind of efficacy is not only corroborated by the persistence of HLS Earth over billions of years, but also mirrors HLS evolution towards increasing ecological complexity as a result of consecutive passages through adaptive cycles. Such cycles appear to have been clocked by terrestrial and/or cosmic catastrophes, inciting species mass extinctions and organizational disorder as breeding grounds of newly consolidating and increasing diversification, sensu Ω-α-r phase transitions (Lenton and Watson 2011). The *program* sensu PICERAS of HLS Earth is encoded in the interaction systems of the major recycling loops, stabilizing the chemical quasi-equilibrium (e.g., carbon or nitrogen cycle, Lovelock 1979).

The persistence and even flourishing of life despite (or because of) repeated Earth-historical catastrophes proves sustained energetic and material self-regulation at HLS Earth level. Absence of self-regulation would have meant progressive decline in species number after each of the extinction events. Instead, species number at least recovered or increased between mass extinctions, mostly accompanied by ecological niche diversification and, hence, evolutionary HLS advancement. Mass extinctions, no matter by what reasons, act as global Ω-phases and apparently are part of self-regulatory HLS Earth-level mechanisms. Not only life is sustained from fragmented but conserved remnants (as some kind of global-scale memory), but life is rendered also, during subsequent α and r-phases, more robust, i.e., becoming buffered against further biogeochemical impacts. The panarchy of HLSs constituting HLS Earth guarantees *adaptability* and *seclusion* (according to the PICERAS concept; see above).

How do species mass extinctions consolidate HLS Earth? Impacts causing mass extinctions disrupt the usually gradual, regular course of evolution, quasi "panic-like" shaking up evolutionary "boredom," so that currently fittest life forms suddenly are not successful anymore. Conversely, the "luckiest" life forms, which coincidentally rather than by long-term adaptation are able to cope with overthrown environmental scenarios turn out as "winners." As the "losers" open space and other resources to the "winners," these then fill re-opened or newly created ecological niches through adaptive radiations based on conducive pre-adaptations (cf. Sect. 3.4). Examples are favored mammals and birds upon the Cretaceous catastrophe together with the intensifying angiosperm diversification, the similarly performing evolution of gymnosperms and reptilians past the Permian and Triassic extinctions, or the onset of multicellular life or manifold aerobe, energetically efficient microorganisms upon global glaciations. Major catastrophes such as mass extinctions always come with a certain, although low probability. The longer a system prevails, the more likely such events occur. Evolving systems operate at self-organized criticality (Bak 1999). Life on Earth has demonstrated *improvisation* (sensu PICERAS) in dealing with these major catastrophes.

Obviously, the probability of such large-scale events to leave a major impact closely relates to the system's current resilience. With an increasing maturity of internal organization, i.e., connectedness, and perturbations as being naturally encountered at any time, causal reaction chains may be triggered, which cause the system to undergo major change. The Corona pandemic provides a convincing example. With global economies and international trade-systems being optimized by globally acting enterprises, the spread of a new virus in parts of the Earth eventually challenges the economy on a global scale, since chain reactions of infection events allow the virus to spread over much larger distances in short time. Such avalanches of reaction events have been shown to explain system breakdown in many examples, providing a mechanism for explaining self-organized criticality (see Bak 1999). Therefore, reaction cascades initiated by small-scale, conventional perturbations foster *regeneration* via major catastrophes, when increased connectedness sufficiently reduced resilience of the HLS. Summarizing, the seven PICERAS conditions can be identified for HLS Earth, leading to the conclusion that HLS Earth is subject to evolutionary adaptation via sequential selection.

The trend of increasing ecological diversification, functional specialization and, hence, environmental adaptiveness of life forms, resulting from a prolonged sequence of mass extinctions, affirms self-regulation and advancing evolution of HLS Earth as a whole. Hence, HLS Earth evolves without resource-oriented competitors by proceeding through adaptive cycles, as reflected by any subordinate HLS. Adaptive cycling of HLS Earth is determined by mass extinction events (creativity phases) and interim time intervals (consolidation phases). The sustained self-regulation in flux control of HLS Earth apparently enables biogeochemical co-evolution towards self-containment of life as being buffered by ecological complexity. Relativizing the role of competition in evolution implies to challenge competition-driven selection as a seemingly non-disputable conviction or dogma (Kimura 1983; Scherer 2017). Supported is the challenge by augmenting empirical evidence about manifold evolutionary processes, whereas the theory of evolution still is underdeveloped and impedes ecologically mechanistic clues about how life differentiation progresses on Earth.

However, what about the dramatic species mass extinction accompanied by Global Change and climate warming as currently incited by Man, leading to species losses that overtop those of previous extinctions while proceeding much more rapidly? Will the current resource-exploitive stage in Earth history, termed Anthropocene, become that extensive, so that self-regulatory capacities of HLS Earth may fail to preserve life, including mankind (Lüttge and Scarano 2019)? As self-regulation of HLS Earth has proven reliable over eons and several mass extinction catastrophes, life as such seems unlikely to become erased by Man. Conversely, only cosmic catastrophes such as planetary collisions, gravity effects on Earth's orbit by passing nomad planets, gamma flashes emitted from hypernovae or ageing Sun when entering the red giant stage, may possess capacities of ultimately eradicating all life, i.e., destroy HLS Earth (cf. Ward and Brownlee 2004a). Notwithstanding, we should not become self-confident in view of Earth-historical resilience capacities experienced so far. As life as such may not be doomed by Man, it may be mankind by its collapsing interference-prone civilization, which is both directly and indirectly menaced by the anthropogenic impacts. Such risk is grave, as civilization-determined lifestyles have lost their anchoring in Man's evolutionary origins. In preventing mankind to perish, research on biogeochemical settings and resilience capacities of HLS Earth is to be intensified for identifying such crucial tipping points in the global interaction network, which may inevitably lead to human disaster upon exceedance.

5 Conclusions

Starting with the holobiont sensu stricto as one kind of HLS, a central host organism together with its interacting associated microorganisms forms the entity of selection and (co-)evolution. Such kinds of functional aggregation are encounterable at any hierarchical scale of ecological organization, ranging from cells with endosymbionts up to HLS Earth as the two extremes, hence, challenging conceiving evolution as an invariant attribute of any scale across the entire hierarchical scope. In adopting the challenge, we realize, within our perspective as observers, commonalities of evolutionary mechanisms across all kinds of HLS irrespective of ecological scale, as outlined in this essay. HLS principles are not only encountered at the organismic and sub-organismic scale including holobionts sensu stricto, but also at ecosystem level integrating its subordinate holobiontic HLSs, and even at the scale of living Earth as the hierarchically uppermost and globally comprehensive ecosystem.

Across scales, HLSs are subject to evolution as a fundamental feature of life, shared with all life systems as definable by observers. At hierarchically low ecological scales, evolution is readily conceivable as operating through Darwinian and neo-Darwinian principles. However, at high scales, beyond holobionts sensu stricto, visualization of HLS evolution is demanding, given that competition and reproduction are conventionally seen as evolutionarily intrinsic and organism-focused features, ultimately anchored in DNA-encoded information. As such latter requirements are not readily recognizable in their absoluteness at the scales of ecosystem-level HLSs and, not at all, of HLS Earth, selection, heredity, and adaptability are to be viewed in generic terms beyond DNA-focused scopes, without violating fundamental criteria indispensable to all kinds of life. In fact, generic qualities are assessable regarding mutation, inheritance, adaptability, and selection, reaching beyond genome levels and organismic generation intervals. It was demonstrated in this essay that the generic qualities are substantiated during passages of HLSs through adaptive cycles, without requiring resource-related competition or synchronization with organismic generations. In such ways, informational entities as integrals of interaction networks and agents are transferred as "songs sung" over time independently of "singers," i.e., particular agents, which principally are renewable and exchangeable. On such grounds, evolution proceeds at any scale, including ecosystem HLSs and HLS Earth. Nevertheless, unrestrictedly valid are the underlying Darwinian principles of mutation, heredity and selection as related to adaptability, which are not violated under generic views, but exceed the narrow scope of gene-centrism.

The joint evolutionary challenge across HLSs, irrespective of hierarchical position, is keeping pace with the perpetually altering energy and matter exchanges within environments. Met is the challenge through ongoing self-regulatory processes. These are mediated by network-immanent feedback control. Otherwise, HLSs would fail in consolidating, at least for transitory periods of time (as experienced by observers), and disintegrate towards thermodynamically determined randomness. Ultimate optima in self-regulation stay unreachable, given the continually and unpredictably fluctuating biogeochemical settings. In such terms, the HLS concept is the substantiation of empirically assessable cause-effect relationships, generating complex multi-level interaction networks, which are anchored in ecologically, developmentally, and evolutionarily cross-linked mechanisms (EvoDevoEco principles).

Thus, discussing HLS evolution obviously leads us to beyond neo-Darwinisms. However, it is vividly debated if we really need a new second synthesis of evolution theory sensu Laland et al. (2014), or if the aspects outlined above are not intrinsic already to evolutionary theory, but overlooked, without requiring further elaboration (Wray et al. 2014). One may argue that rather than pursuing abstract debates about appropriate viewpoints, one may consider the "stream" of knowledge to be continuously fed and up-dated by "confluents" of new evidences, concepts, and re-thinking (Fig. 21 in Chapter 20 of Lüttge et al. 2010). If evolution means survival-supportive adaptation in a broadest sense (as based on the range of mechanisms highlighted above), sequential selection fosters survivability under the perpetually changing environment via valuing consecutive, but variable states or phases in functionality.

Treating HLSs as integrated co-evolutionary units (Sect. 2), the various forces acting on them during adaptive cycles incite dynamics that drive evolutionary progress through EvoDevoEco mechanisms (Sect. 3). Such principles are also involved, whenever biogeochemical settings of HLS Earth change, which permanently has been the case throughout Earth history. Major changes of its biological face during the Earth's history were shaped by plate tectonics modifying the position and configuration of the continents (Carroll 2000). Dramatic episodes of change, for example, were the global snow-slush phase at 750 to 575 million years ago, when Earth was almost completely covered by a thick crust of ice. Or later-on, when rainforests largely expanded throughout the tropics of the Carboniferous era at 360 to 290 years ago. Or, when at times several devastating waves of species mass extinctions perturbed HLS Earth evolution such as the one 65 million years ago, which terminated the era of dinosaurs, but boosted mammal, bird, and angiosperm differentiation and proliferation, and, accordingly, ecological niching (Lenton and Watson 2011). All such eons and events were part of continuous adaptive cycling of HLS Earth as passing through altering biogeochemical settings, providing dynamic grounds for sequential selection, while paving the way for evolution (Sect. 4.2). The emerging adaptive advancement of HLS Earth up until today mirrors successfully functioning heredity of acquired biogeochemical traits and evolution void of discrete organismic generation intervals or resource-focused competitive influences.

To conclude, any HLS, including HLS Earth, evolves as continually complying with, i.e., adapting to, shifts within the internally and externally encounterable, biotic–abiotic interaction network, as otherwise persistence is doomed to expire. Success in HLS evolution depends, during adaptive cycling, on oscillations between internal rebuilding ("informational creativity") and modified continuation ("informational consolidation"), implying re-arrangement with the dynamically changing biogeochemical settings through trial-and-error principles. Although adaptive cycles may be infiltrated or replaced by such concurrent HLSs, resource-related competition is not mandatory to cycling and, hence, HLS adaptations during evolutionary advancement (Sect. 4), whereas competition sensu urge to continuously adapt remains an essential factor for selection. Overall, HLSs without being ruled by restrictive neo-Darwinian definitions are units of evolution.

6 Summary

Research has unraveled manifold interrelationships between highly evolved organisms and their associated microorganisms, functionally tied together through co-evolutionary, mutually beneficial advancement towards emerging synergistic properties. The association of a central host, i.e., Higher Plant or Animal, and its microbial companions is termed holobiont, the functional core of which is constituted by a multiple interaction network and understood as unit of selection. As holobiont–intrinsic interaction patterns also prevail at hierarchically subordinate and superior scales of ecological organization, any living association forming an interaction network can be called holobiont-like system (HLS), with the holobiont sensu stricto being one specific case. Rather than being host-centric, HLSs in general comprise equitably interacting agents. Notwithstanding, the question arises, in particular, at the high-ranking scales of ecosystems or the biosphere, as to whether respective HLSs obey fundamental principles of life and, hence, also of evolution. How to conceive evolution, being anchored in a Darwinian sense in mutation, heredity and selection, and conventionally viewed under gene-centric perspectives, however, if considering HLSs at ecosystem and biospheric scales? This essay strives for clarification.

The exploration begins with assessing the ecological settings and scales of HLSs and their consistency with life criteria, as intrinsically based on organizational and regulatory capacities of self-containment. Coercively, the history of evolution theory is elucidated, revealing conceptual shortcomings, which largely arise from DNA-restricted interpretations of evolutionarily relevant processes, when fathoming HLS evolution at high ecological scales. Derivable from Darwinian principles, HLSs are discernable, however, as units of selection. Key is to ascribe generic qualities to mutation, heredity, and selection by widening DNA-encoded information to such additionally stored in structural and functional HLS properties as acquired upon historical contingencies. Such informational properties are dynamics in species and occupied niches diversity or, more generally, fluctuations within HLS-level interaction networks, being realized through co-evolutionary principles as anchored in integral "EvoDevoEco" mechanisms.

On such grounds of informational variation (generic mutation), memory (generic encoding) and temporal transfer decoupled from generational sequences (generic heredity), HLS evolution proceeds through repeated adaptive cycling. Cycles are divisible into alternating periods of HLS predictability upon resource exploitation and temporarily gained informational accumulation (heredity phases r and K, respectively) versus stochasticity determined by resource release and enabled informational reorganization (mutation phases Ω and α, respectively). In essence, each turn varies HLS properties under the perpetually fluctuating environmental settings, constituting evolutionary advancement. The latter is based on generic selection as an autonomous process, examining HLS adaptability by differentially favoring emerging variants. Such kind of evolutionary evaluation process implies a more general understanding of competition, capturing the challenge of HLS to maintain adaptability (sensu adaptively competing with progressive change). Hence, no other HLSs are required as competing for resources, as any interaction scenario shapes HLS performance and offers targets to sequential selection during recurrences of adaptive cycling. That conclusion is important in view of the biosphere, i.e., HLS Earth.

HLS Earth has run through its adaptive cycles and, hence, evolution since the onset of life at about 3.5 billion years ago, being shaped through biogeochemical developments and irreversibly stored and traded contingency effects of, e.g., volcanism, tectonics, biogenic O_2 enrichment, marine and terrestrial life advancements or cosmic impacts. Species mass extinctions upon biogeochemical catastrophes fostered rather than limited niches diversification and biodiversity towards steadily increasing ecological complexity, vividly affirming evolutionary progress of HLS Earth as a whole. Mass extinctions apparently acted as pulse generators of adaptive cycling, creating phase α-r transitions with emerging modified variants of HLS Earth. Such HLS modifications in adaptability to quaked environments have resulted in HLS Earth as encountered today upon passing the continual valuing scrutiny of sequential selection.

In conclusion, HLS evolution becomes perceivable, if leaving neo-Darwinian gene centrism. Evolution in a broadest sense arises from a wide range of mechanisms beyond DNA scopes, crucially from sequential generic selection of variants in HLS performance during adaptive cycling under the changing environment. HLS phases oscillate between “informational creativity and consolidation” as subjected to trial-and-error principles, while evolving without necessarily requiring resource-focused competitive drivers.

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Plant Proteolysis in Development: Insights and Functions

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Abstract Plant proteolysis is an important process through which proteome quality and quantity surveillance is accomplished. Proteases regulate protein turnover and expand their functional diversity. Proteolysis is mostly known as a means to remove proteins but may also result in the production of new shorter proteins. Hence, proteolysis is directly linked with many aspects of the plant's lifecycle. Here, we provide an overview of selected examples of the major proteolytic pathways known, digestive (autophagy, proteasome, ubiquitin-like pathways and N-end rule) and limited proteolysis in development.

Keywords Autophagy, Development, NEDD, N-end rule, Proteases, Proteolysis, SUMO

1 Introduction

In plants, cellular and extracellular proteomes show remarkable functional flexibility, a result of different posttranslational fates, including a broad range of covalent modifications (Liu et al. 2020). Almost all proteins, regardless of their lifetime, biochemical properties and cellular function, are sooner or later subjected to proteolysis (Liu and Moschou 2018). The term "proteolysis" is erroneously associated usually with protein end-point degradation and thus loss-of-function. However, proteolysis is like a Swiss-knife and has many trades. Indeed, each protein thus has multiple proteolytic routes on which to embark, with proteolysis outcomes varying between two extremes: either complete degradation (digestive proteolysis) or specific cleavage of the polypeptide chain at one or a few sites (limited proteolysis). While the outcome of digestive proteolysis is usually protein destruction and functional loss, limited proteolysis can additionally lead to the maturation of proteins or peptides and gain- or switch-of-function (Liu and Moschou 2018). The substrate and products of many proteolytic modules are known; however, the corresponding protease remains unidentified. By contrast, many proteases have unknown substrates (orphan proteases).

In this chapter, we discuss proteolytic aspects with direct links to development. We also provide an overview of biological pathways that utilize specific proteolytic cleavage as a mechanism for tuning their outcomes. Due to the immense amount of data on the chapter's topic, our discussion is succinct and by no means exhaustive. We thus occasionally refer the interested reader to excellent reviews that provide deeper insights.

1.1 The Basics of Limited and Digestive Proteolysis

While all proteins give in to proteolytic degradation, the proteome fraction subjected to limited proteolysis remains elusive. Often regarded as a mere degradative mechanism in the destruction of proteins or turnover in maintaining physiological homeostasis, recent research in the field of degradomics (i.e. the high-throughput study, usually by proteomics, that can detect proteolysis products and even cleavage sites) has led to the recognition of two main yet unexpected concepts. First, that targeted proteolytic cleavage events by a wide repertoire of proteases are pivotal regulators of most, if not all, developmental processes. Second, an unexpected *in vivo* abundance of stable cleaved proteins revealed pervasive, functionally relevant protein processing.

Below, we succinctly describe the two main proteolytic branches. As of note, as the C-end rule pathway has only recently emerged as a potential proteolytic branch and is as yet unknown in plants, we refer interest readers to, e.g., Lin et al. (2018).

1.2 Digestive Proteolysis

1.2.1 Autophagy

Autophagy carries out digestive proteolysis and usually requires endoproteases for full functionality. Autophagic protein degradation takes place in the vacuole and can be either bulk or selective, i.e. when certain cellular components are preferentially targeted for destruction (Fig. 1a; (Minina et al. 2017a)). Selective autophagy is determined by the interaction of ATG8 with specific cargoes regardless of their size. Readers may refer to Marshall and Vierstra (2018), which is an overview regarding selective autophagy. As of note, the repertoire of autophagy extends beyond proteolytic degradation; it also digests nucleic acids, lipids and carbohydrates but here we will focus on proteolysis.

Autophagic degradation is carried out via recognition of substrates by specific adaptors and their sequestration into a double-membrane vesicle, the autophagosome, which is then delivered to and degraded in the vacuole (Minina et al. 2017a). Autophagy-related (ATG) proteins are posttranslationally activated and modulate autophagosome biogenesis ((Marshall et al. 2019) and the references therein). Interestingly, autophagy is regulated by limited proteolysis that takes place in the early stages of the autophagy pathway. In particular, the small ubiquitin (Ub)-like protein ATG8 is cleaved C-terminally by the cysteine protease ATG4 at a highly conserved Gly and the exposed C-terminal glycine is conjugated with the growing autophagosomal membrane (Yoshimoto et al. 2004; Pérez-Pérez et al. 2021). This residue coincides with the penultimate residue in yeast, while in most ATG8s this conserved Gly does not occupy this position. ATG8 is then released from the mature autophagosome membrane by the same ATG4 protease (Pérez-Pérez et al. 2021).

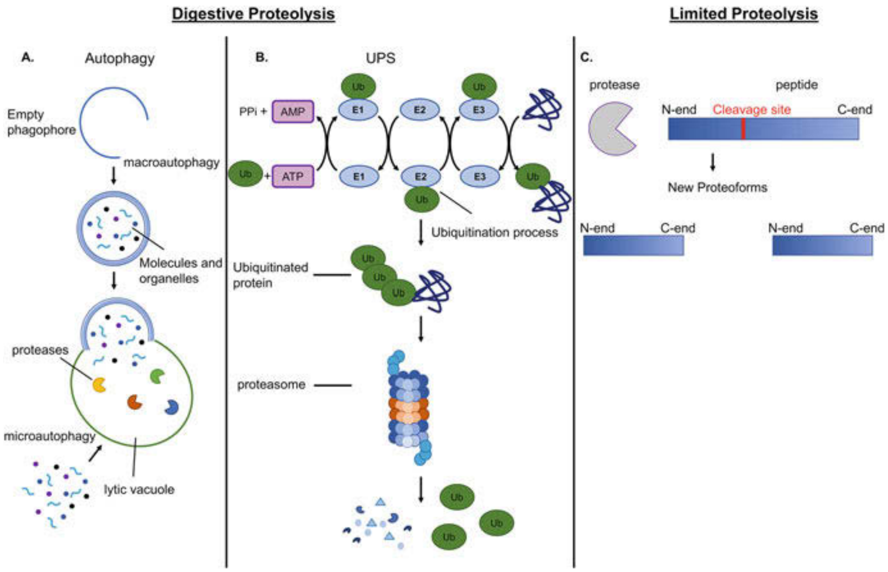

Fig. 1 Schematic representation of the major pathways in digestive and limited proteolysis. (**a**) Autophagy: Molecules and organelles are either enclosed in autophagosomes (macro-autophagy) or enter directly the lytic vacuole (micro-autophagy). (**b**) Ubiquitin-Proteasome System: Proteins are selectively ubiquitinated and recognized by proteasome leading to full degradation. (**c**) Limited proteolysis: Proteases recognize and cleave their protein-targets, creating new proteoforms with different properties such as new interactions or subcellular localization. Figure was created using BioRender (https://biorender.com)

1.2.2 Proteasome

Unlike autophagy, the Ub-proteasome system (UPS) is almost always selective degrading only Ub-tagged proteins. In the UPS pathways, Ub covalently conjugates to substrate lysines (K) through activating (E1), conjugating (E2) and ligating (E3) enzymes (Sadanandom et al. 2012; Santner and Estelle 2010; Spoel et al. 2009; Eckardt 2001; Emenecker et al. 2020; Lin et al. 2020). Ub is first activated by E1 and then transferred onto an E2 conjugating enzyme. Subsequently, E3 Ub-ligases interact simultaneously with a Ub-loaded E2 and the substrate and mediate the formation of isopeptide bonds between the Ub C-terminus and the acceptor K. Depending on the Ub modification nature (e.g. K48 or K63), Ub-carrying proteins have different fates, two prominent examples of which are degradation by the UPS (K48-linked Ub chains) and endocytosis from the plasma membrane to the vacuole (K63-linked chains) (Schwechheimer 2018). The single-subunit E3 ligases include proteins containing a conserved REALLY INTERESTING NEW GENE (RING) domain, a U-box domain, or a Homologous to the E6-AP Carboxyl Terminus (HECT) domain that mediates interaction with the E2-Ub.

1.2.3 Ubiquitin-Like Pathways

Other posttranslational modifications (PTMs) may associate with digestive proteolytic pathways. For example, an analogous enzymatic cascade to Ub is protein tagging via isopeptide bonds with SUMO (small Ub-like modifier) (Srivastava et al. 2016; Yates et al. 2016a, b). Other Ub-like modifiers (UBLs) include NEDD8 (NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWN-REGULATED 8) or RUB1 (related to Ub 1) (Schwechheimer 2018), URM1 (Ub-related modifier-1) (Wang et al. 2019), ATG8/12 (autophagy 8/12), MUB (membrane-anchored Ub) (Dowil et al. 2011), UFM1 (Ub-fold modifier-1) (Sasakawa et al. 2006) and HUB1 (homology to Ub-1) (Downes and Vierstra 2005). Like in the case of Ub, UBL conjugation generally depends on an E1-E2-E3 cascade and targets the α-amino acid group of K residues. Although UBLs share a similar fold, their functions and properties differ from Ub. Typically, only a small fraction of a protein competent for SUMOylation is usually SUMOylated, while proteases may execute de-conjugation reactions, including those of the posttranslationally formed isopeptide bonds found in mono-, multi- and polyUb chains or the very similar small SUMOs (Yates et al. 2016b) and conjugates (Schmaler and Dubiel 2010).

In plants as well as in other eukaryotes, the number of E3 ligases for NEDD8 and of NEDD8-modified proteins is much smaller when compared to Ub-modified ones (Hakenjos et al. 2013). Little is known about the function or conjugation of UFM1, MUB and HUB1 in plants. While knowledge of UFM1 is completely missing in plants, all E1, E2 and E3 enzymes for UFM1 conjugation have already been characterized in mammals (Daniel and Liebau 2014). MUB, a UBL present in animals, fungi and plants, is anchored to the membrane owing to the presence of a prenylation signal at its C-terminus instead of the usual di-glycine motif (Nagels Durand et al. 2016). In yeast and mammals, HUB1 functions non-covalently and no E1-E2-E3 cascade has been identified.

1.2.4 N-End Rule Pathway

The N-end rule relates the *in vivo* half-life of a protein to the nature of its N-terminal (Nt)-residue, which, alongside other requisite features (an unstructured, exposed N-terminus and accessible downstream K), forms a degradation signal called the “N-degron”. Proteins are produced with a methionine (Met; or formylmethionine, fMet) at their Nt (Xu et al. 2015; Frottin et al. 2006; Linster et al. 2015). In most proteins, Nt-Met is cotranslationally cleaved by METHIONINE AMINO-PEPTIDASES (MetAPs), exposing new Nt-residues. The N-end rule pathway has been co-opted to the UPS, targeting proteins for destruction by the UPS through conjugation of a polyUb chain (Zhang et al. 2017a; Gibbs et al. 2011, 2014; 2016; Gibbs 2015). N-degrons are typically conditional, exposed and recognized by Ub-E3 ligases (N-recognins) only under certain conditions.

We know two divisions of the N-end rule pathway: (1) the arginylation (Arg/) N-end rule, which recognizes substrates with unmodified basic or hydrophobic residues and (2) the acetylation (Ac/) N-end rule, which targets proteins bearing certain Nt-acetylated residues (Zhang et al. 2017a; Holman et al. 2009; Vicente et al. 2017). The confirmed "N-recognins" of the Arg/N-end rule: PROTEOLYSIS1 (PRT1) and PRT6 bind to substrates bearing aromatic or basic Nt-residues, respectively (Graciet et al. 2009; Garzon et al. 2007). During protein synthesis, the α-amino group of Nt-residues can be cotranslationally acetylated by ribosome-associated Nt-acetyltransferases (NATs) (Varland et al. 2015). This acetylation occurs either directly on Nt-Met or the 2nd residue after Met-removal by MetAP. Three NATs (NATA, B and C) catalyse the majority of these modifications, with each having distinct substrate specificities. Posttranslational Nt-acetylation also probably occurs.

NAT loss-of-function mutations cause growth defects and reduced photosynthetic efficiency (Gibbs 2015). The NatB loss-of-function mutant *tcu2* shows that Nt-acetylation regulates flowering time and leaf, inflorescence, flower, fruit and embryonic development. Furthermore, recent evidence shows that the N-end rule pathway is linked to meristematic activity in the shoot apical meristem in *Arabidopsis*. The molecular mechanism relates to the degradation of the MicroProtein LITTLE ZIPPER2 (ZPR2) which is degraded by the oxygen-dependent N-degron pathway and thus is stabilized at low O_2 levels (Weits et al. 2019). Key to the hypoxia-mediated regulation are the ERF-VII transcription factors (White et al. 2017). Similar to the aforementioned ZPR2 in the shoot meristem, the O_2-dependent N-end rule pathway degrades the ERF-VII factors which are thus stabilized at low O_2 (Gibbs et al. 2011). As explained below, limited proteolysis depends on a cohort of proteases (Liu et al. 2020; Tornkvist et al. 2019; Liu and Moschou 2018), which may also generate variation in protein turnover by exposing new Nt that can undergo various modifications or amend activities and functions by removing regulatory domains. An example is the BIG BROTHER protein described in Sect. 4.

1.3 Limited Proteolysis

Limited proteolysis is executed by endoproteases which cleave proteins in internal sites, while exoproteases (amino- or carboxypeptidases) trim protein ends (Willems et al. 2017). Proteases enzymatically hydrolyse peptide bonds, resulting in a widespread, irreversible posttranslational modification of the protein's structure and biological function (Fig. 1c). *Arabidopsis* and rice (*Oryza sativa* sp.) have close to 800 putative proteases, classified into clans and families. Proteases cleave peptide bonds by polarizing a normally unreactive carbonyl group of the substrate. The carbonyl oxygen is stabilized in an oxyanion hole, making the carbon atom more vulnerable to attack by an activated nucleophile. Proteases are classified by catalytic type and, at present, these are grouped into those in which the activated nucleophile is a side-chain of an amino acid on the protease (“protein nucleophiles”) and those in which the nucleophile is an activated water molecule (“water nucleophiles”).

Protein nucleophiles can be serine-, threonine- or cysteine-type, while water nucleophiles can either be amino acids sidechains (aspartates or glutamates) or by metal ions bound by sidechains. The corresponding catalytic types of water nucleophiles are known as aspartyl-, glutamyl- or metallopeptidases. Furthermore, some proteases are orphan as their catalytic type remains either unknown or does not fall within any known category. Proteins not annotated as proteases may execute proteolysis. For example, in humans, the enzyme O-linked β-N-acetylglucosamine transferase (OGT) that has a canonical function in serine and threonine glycosylation also executes proteolytic maturation of the cell-cycle regulator host cell factor-1 (HCF-1) (Lazarus et al. 2013). Besides, not all proteases cleave proteins. For example, the *Arabidopsis* γ-glutamyl-transferase 1 and 2 (GGT1 and GGT2) hydrolyse the tripeptide glutathione, some members of the M20 protease family hydrolyse auxin-amino acid conjugates (Bartel and Fink 1995; Davies et al. 1999; Martin et al. 2007), while some carboxypeptidases show acyltransferase activity (sinapoyl-Glc accumulator 1 and 2) (Fraser et al. 2007). Recent evidence suggests that in animals the vast majority of deubiquitinases (i.e. proteases removing Ub from proteins) display isopeptidase and esterase activity (De Cesare et al. 2021). This finding is important, as many esterases could potentially also cleave proteins. We expect that this list of proteases that cleave other than peptide bonds will significantly expand soon.

Among other processes, diverse inhibitors regulate proteases, keep them inactive and, in many cases, a single protease may have more than one inhibitor, while the inhibitors may target more than one protease (Kumar et al. 2015). For example, the *Arabidopsis* inhibitors Serpin1 and disulfide isomerase-5 both interact with and inhibit the cysteine protease RESPONSIVE TO DESICCATION-21 (RD21) (Lampl et al. 2010). The interaction with disulphide isomerase-5 keeps RD21 inactive and it also functions as a chaperone, escorting RD21 en route from the endoplasmatic reticulum via the Golgi to vacuoles (Ondzighi et al. 2008). The acidic vacuolar environment activates RD21, which then contributes to protein degradation in senescing leaves (Otegui et al. 2005; van der Hoorn and Kaiser 2012). Such complex relationships between proteases and inhibitors create complicated proteolytic networks. This is further perplexed by the different inhibitory types: suicidal or reversible.

1.4 The Interplay Between Proteolytic Pathways: The Case of Autophagy-Limited Proteolysis

The best example of the interplay between limited proteolysis and autophagy is ATG8 cleavage by the cysteine protease ATG4. ATG8 is a small Ub-like protein, essential for the elongation and closure of the autophagosomal membrane (Yamasaki et al. 2020). Upon induction of autophagy, ATG8 undergoes limited proteolysis followed by reversible lipidation. The C-terminus of ATG8 is cleaved by the protease ATG4 and the nascent C-terminal glycine is conjugated with a lipid phosphatidylethanolamine (PE) moiety via an amide bond, anchoring ATG8 in an autophagosomal membrane. Intriguingly, ATG8 is removed from the membrane of a mature autophagosome by the same ATG4 protease, which can hydrolyse the amide bond between ATG8 and PE. This ATG4 dual function is crucial for the formation and elongation of the autophagic membrane, proper localization and recycling of ATG8, and finally removal of ATG8 from the mature autophagosomes, which seems necessary for their subsequent fusion with the vacuole (Pérez-Pérez et al. 2021).

ATG4 is the only protease identified among known ATG proteins and is called also "autophagin" (Ding et al. 2018; Rawlings 2013; Vizovišek et al. 2018; Minina et al. 2017a). The balance between proteolytic and delipidating ATG4 activities defines ATG8-PE amount present in the cell. Unlike yeast, most of animal and plant genomes contain several orthologs of ATG4 and ATG8 genes, indicating potential sub- and/or neofunctionalization. To our knowledge, until very recently, only mammalian ATG8 orthologs had been shown to have different roles in certain types of selective autophagy (Lystad et al. 2014). While plant ATG8 orthologs have not yet been sufficiently characterized, assessment of the specificity of two autophagin orthologs towards nine ATG8s in *Arabidopsis* has revealed only slight selectivity but differences in their proteolytic activity and sensitivity (Seo et al. 2016). Moreover, recent advances have shown the involvement of different isoforms of ATG8 specifically in the regulation of selective autophagy pathways in plants (Zess et al. 2019).

Calpain-dependent cleavage of ATG5 (Yousefi et al. 2006) or caspase-dependent cleavage of ATG6 (Wirawan et al. 2010) in mammals yields protein fragments that cannot sustain autophagosomes formation, but instead activate apoptotic cell death in animals. N-terminal domain of the catalytically inactive protease ATG4D leads simultaneously to activation of the ATG4D and to release of the cytotoxic C-terminal domain of the protein (Betin and Lane 2009). Thus, limited proteolysis of these proteins switches cellular stress response from survival to death. Likewise, in *Arabidopsis* cleavage of the plant Bcl2 associated athanogene 6 (BAG6) by the aspartyl protease APCB1 induces autophagosome formation and restricts necrotic lesions spreading induced by the necrotrophic fungus *Botrytis cinerea* (Li et al. 2016b). Another example of an interplay between limited proteolysis and autophagy in plants is provided in Sect. 2.2.

2 Proteolysis in Reproductive Development and Embryogenesis

2.1 Reproductive Development

Flowers are the reproductive plant organs. Reproductive development is characterized by major metabolic changes. Through meiosis and mitosis, the male and female gametophytes also called the pollen grain and the embryo sac, respectively, are generated from anther tissue and ovules. Furthermore, a very important process for successful reproductive development is pollen germination followed by tube growth. This process is relatively fast and dynamic, characterized by vesicular trafficking and cytoskeletal changes, as well as highly active metabolism (Cameron and Geitmann 2018). These changes necessitate the use of proteolytic systems to remodel the proteome and thus it is hardly surprising that limited and digestive proteolysis regulate reproductive development.

The cysteine protease separase (known also as "Extra Spindle Poles", ESP) executes the release of sister chromatid cohesion during meiosis and mitosis (Yang et al. 2009). ESP-dependent proteolytic cleavage of the α-kleisin subunit of the cohesin complex that holds sister chromatids together at the metaphase-to-anaphase transition is essential for the proper segregation of chromosomes (Minina et al. 2017b; Cromer et al. 2019). In *Arabidopsis*, meiotic-specific ESP RNA interference blocked cohesin removal from chromosomes and resulted in the presence of a mixture of fragmented chromosomes and intact bivalents leading to alterations in nonhomologous centromere association as well as disruption of the radial microtubule system after telophase II. Moreover, ESP RNA interference affects the proper establishment of nuclear-cytoplasmic domains, resulting in the formation of multinucleate microspores. Likewise, two *Arabidopsis* putative glycosylphosphatidylinositol (GPI)-anchored aspartic protease genes, A36 and A39, which are highly expressed in pollen and pollen tubes, play a role in reproductive development (Gao et al. 2017a, b). a36 a39 mutants show precocious cell death of pollen and are female gametophytic defected. GFP-A36 and A39 localize at the plasma membrane and cytoplasmic puncta, colocalizing with the GPI-anchored protein COBRA-LIKE10 which plays a role in the cell wall structure and pollen tube guidance (Li et al. 2013). Hence, in *a36 a39*, the abundance of highly methyl-esterified homogalacturonans and xyloglucans is significantly increased in the apical pollen tube wall.

Aspartic proteases also play vital roles in tapetum degeneration timing, which is crucial in sexual reproduction (Olsson et al. 2019; Yang et al. 2012; Cecchetti et al. 2008). In *Arabidopsis* and rice, the aspartic proteases AtUNDEAD and OsAP25/OsAP37, respectively, modulate tapetal PCD timing; their absence leads to pollen abortion (Phan et al. 2011). In rice, S5 participates in indica-japonica hybrid fertility and could stimulate ER stress, giving rise to PCD in the embryo sac (Chen et al. 2020; Yang et al. 2012). OsAP65 is essential for pollen germination and tube growth (Huang et al. 2013). In *Arabidopsis*, loss of function of the ER-localized

PROMOTION OF CELL SURVIVAL1 (PCS1) aspartic protease causes gametophytic degeneration (Ge et al. 2005). Therefore, plant aspartic proteases may be implicated in the restriction of PCD in plant reproduction, although the underlying mechanism is not clear.

The MMS21 (HPY2) is a SUMO E3 ligase conserved in eukaryotes and required for DNA repair and chromosome integrity maintenance. In *Arabidopsis*, MMS21 loss-of-function causes defective meristems, dwarf phenotypes and gametophytic defects (Liu et al. 2014). SUMO E3 ligase MMS21/HPY2 represses the transition from the mitotic cycle into the endocycle in *Arabidopsis* (Ishida et al. 2012). The *hpy2-1* mutant survives for only a few weeks under normal growth, but a few seedlings eventually form shoots that show fasciation and defects in phyllotaxis. Their root meristems contain abnormally enlarged cells and a higher proportion of cells in the endocycle. These endocyclic cells also contain higher DNA content (reaching 64C and 128C) and much larger nuclei compared to wild-type. Mutants of MMS21/HPY2 that survive through the reproductive stage display severe fertility defects exemplified by a much-reduced seed set and increased rate of seed abortion (Ishida et al. 2012; Liu et al. 2014). Reciprocal pollination experiments suggested that most of the reduced fertility of *hpy2* mutants is likely due to pollen defects, although some defects in female gametophyte may also contribute to sterility but to a lesser extent. These and other experiments also suggested that *hpy2* pollen tube growth was defective even in wild-type pistils. These results are consistent with MMS21/HPY2 expression in anther and pollen. Further characterization revealed that *mms21/hpy2* anthers have morphological defects, are generally variable in size and shape and produce fewer and nonviable pollen grains compared with wild-type (Liu et al. 2014). Collectively, these results suggest that MMS21/HPY2 is required for male gametophyte development.

Regarding flowering, the SUMO ligase (*siz1*) and SUMO protease (*esd4*) mutations reduce the floral repressor *FLOWERING LOCUS C* (FLC) mRNA abundance and consequently enhance SOC1 expression which promotes flowering. Both mutants show compromised salicylate (SA) signalling; high SA levels associate with lower mRNA levels and accelerated flowering (Jin et al. 2008). Besides, SIZ1 may also activate FLC expression through an SA-independent pathway that requires the flowering time gene FLD (Jin et al. 2008). On the other hand, the FLC protein interacts with SIZ1, reducing the SUMOylation of FLC *in vitro* (Son et al. 2013). Furthermore, SIZ1 functions did not depend on SA levels because expressing *nah*G in *siz*1 did not restore ovule viability, suggesting that SIZ1 plays a direct role in ovule development. Reciprocal pollination and pollen viability analyses revealed that *siz*1 pollen is normal (Ling et al. 2012). On the other hand, siz1 female gametophyte could not support full fertilization of wild-type pollen and scanning electron microscopy revealed that pollen grains germinate and pollen tubes migrate through the style of *siz1* plants but fail in the final stage of pollen tube guidance to reach the micropylar opening and hence fail to enter the embryo sac. Collectively, these findings suggest that SIZ1 is required for normal female gametophyte development. In the aforementioned cases, however, the role of these proteases in mechanistic terms is unclear.

Pollen tube growth is considered a costly and vigorous process because the cell needs to provide large amounts of the cell wall and membrane components, newly synthesized proteins and energy to fulfil growth demands. As described above, *atg6* causes male sterility due to the lack of pollen germination, but knockdown of this gene leads to morphologically normal pollen with decreased germination rate in comparison with wild-type plants (Fujiki et al. 2007; Harrison-Lowe and Olsen 2008). In rice *Osatg7* and *Osatg9* mutants showed complete sporophytic male sterility and decreased anther dehiscence under normal growth conditions, indicating that autophagy is crucial in reproductive development (Kurusu et al. 2014). Furthermore, pollen of these mutants appeared premature due to defects in anther during maturation, while heterozygous plants have normal pollen. Besides, the pollination of the heterozygous plants with wild-type resulted in normal fertility. These findings suggest that the cause of immature pollen phenotype displayed by autophagy-defective mutants depends on defects in various organs or parental tissue. In accordance, analyses of *Arabidopsis* and maize (*Zea mays*) plants harbouring mutations in the autophagy genes (atg) indicated that autophagy contributes to nitrogen remobilization from vegetative to reproductive tissues, including seeds (Have et al. 2017).

2.2 Embryogenesis

In this section, we focus on seed-carrying plants. In a plant seed, the embryo lies dormant surrounded by nutritive endosperm while awaiting suitable conditions to germinate. A hydrophobic cuticle around the embryo protects it from water loss during the early days of growth. Seeds carry large amounts of seed storage proteins, which serve as the primary source of nitrogen for the growing seedling during germination. In developing dicot seeds, the most abundantly expressed storage proteins are members of the 2S albumin and the 7S and 11S globulin protein families. Precursor polypeptides of these storage protein classes are synthesized at the ER, and the mature (processed) polypeptides of all of these three protein classes accumulate inside specialized vacuoles, called protein storage vacuoles (PSVs) (Delgadillo et al. 2020).

In the developing seed, a bidirectional molecular dialogue between embryo and endosperm safeguards cuticle integrity before germination and involves a limited proteolytic pathway (Doll et al. 2020). In this pathway, the ABNORMAL LEAF SHAPE1 subtilase produces the TWISTED SEED1 (TWS1), a peptide that acts as a GASSHO ligand and is recognized by the GASSHO receptor-like kinases. Cuticle surveillance depends on the action of the subtilase, which, unlike the TWS1 precursor and the GASSHO receptors, is not produced in the embryo but the neighbouring endosperm. Active TWS1 precursor mediates the GASSHO-dependent cuticle reinforcement in the embryo.

Autophagic cell death is considered one of the major programmed cell death (PCD) types in eukaryotes (Bozhkov and Jansson 2007). The most characteristic example of autophagic PCD is the embryonic suspensor rupture. This organ is an early embryonic structure that links the embryo to the endosperm and its role is to mediate the transportation of nutrients and signalling molecules as well as to push the embryo through the endosperm (Peng and Sun 2018). In Norway spruce (*Picea abies*) the cysteine protease metacaspase type II (mcII-Pa) mediates lytic vacuole formation, thereby promoting suspensor death. *mcII-Pa* knockdown results in fewer autophagosomes in suspensor cells, however, the opposite is invalid, indicating that mcII-Pa activates autophagy and not vice versa (Minina et al. 2014; Reza et al. 2018). Moreover, ATG5 and ATG6 gene downregulation disrupts suspensor differentiation leading to alternative necrotic death and arrest of embryonic development. Yet, the molecular mechanism by which *mcII-Pa* modulates vacuole formation is unknown.

3 Proteolysis in Hormonal Regulation

Unlike hormonal regulation in animals, plant hormonal pathways depend on breaking points, i.e. proteins, with the ability to restrict a whole pathway. In this section, we provide examples of limited and digestive proteolysis and their effect on the regulation of development. Noteworthy, we present only a subset of direct functions that have been recently described as key regulators of hormonal signalling. The UPS of key regulatory proteins in hormonal pathways has been demonstrated or is at least likely, for all of the phytohormone response pathways.

3.1 Examples of Digestive Proteolysis in Hormonal Regulation

Here we provide selected examples of digestive proteolysis in hormonal regulation. More detailed discussion on the roles of UPS in hormonal pathways can be found in Kelley and Estelle (2012); Lopez-Obando et al. (2015) and autophagy in hormonal regulation in Gou et al. (2019); Liao and Bassham (2020).

3.1.1 Gibberellins

Plant embryos survive for a long time in a developmentally arrested state as dry seeds (i.e. dormancy), a state rapidly reversed during germination. The plant hormone gibberellin A (GA) promotes germination and dormancy loss when environmental conditions are favourable for germination and growth. GA stimulates plant growth and development by targeting the DELLA proteins for proteolytic degradation by the UPS. DELLA proteins are the key repressors of almost all GA responses. There are five DELLA proteins in *Arabidopsis*, the GA INSENSITIVE (GAI), REPRESSOR OF *ga1-3* (RGA), RGA-LIKE 1 (RGL1) and RGL2 (Van De Velde et al. 2017; Salanenka et al. 2018).

A good example of GA functions is the growth modulation in different light conditions. Light promotes plant photomorphogenesis, giving rise to open and expanded cotyledons, and short hypocotyls in *Arabidopsis* seedlings. In the dark, seedlings undergo de-etiolation (Lyu et al. 2019), characterized by closed cotyledons and elongated hypocotyls (Lyu et al. 2019). A subset of basic helix-loop-helix (bHLH) transcription factors, known as phytochrome-interacting factors (PIFs), has a key role in etiolation and light-regulated plant development. Upon illumination, the photoactivated phytochromes trigger PIFs' rapid phosphorylation and subsequently UPS-mediated degradation, leading to transcriptional changes that promote photomorphogenesis. DELLAs promote PIF degradation through UPS. When GA is present, GA receptor GID1 binds to DELLAs to form GID1-GA-DELLA complex, which then triggers DELLA proteins degradation by the UPS. DELLAs have a conserved DELLA domain (motif Asp-Glu-Leu-Leu-Ala) at the Nt, essential for GA-triggered protein degradation (Li et al. 2016a).

SUMO regulates GA signalling, for example through SLEEPY1 (SLY1) SUMOylation by SIZ1 (Mazur et al. 2019). SLY1 is an F-box protein component of an SCF complex (SCF^{SLY1}) that mediates the interaction of GID1 with DELLA proteins in response to GA, thereby facilitating DELLA degradation (Wang et al. 2009). SIZ1 SUMOylates SLY1, this increases SLY1 stability and interaction with DELLA proteins, promoting growth due to enhanced DELLA degradation (Kim et al. 2015). Interestingly, GA induces SIZ1 expression and stimulates SLY1 SUMOylation. These data propose that GA stimulates growth by inducing SIZ1 expression, which in turn leads to SLY1 sumoylation, stabilization and interaction with DELLA proteins. SLY1 interaction with DELLA leads to subsequent DELLA degradation. This model implicates SUMO as a positive regulator of GA-mediated plant development.

3.1.2 Jasmonates

Jasmonic acid (JA) together with its precursors and derivatives, referred to as jasmonates (JAs), regulate – among other processes – the response to wounding, by inducing specialized metabolism (Nagels Durand et al. 2016). JAs further contribute to plant plasticity by regulating responses to abiotic stresses, thereby adjusting growth and productivity under adverse conditions. Finally, JAs are also important in the regulation of responses to light and development (Zhou et al. 2019).

The core JA-signalling module comprises (1) MYC2, a key transcription factor regulating expression of JA-responsive genes, (2) Jasmonate ZIM-domain (JAZ) proteins, repressors that inhibit MYC2 activity in the absence of the hormone and (3) CORONATINE INSENSITIVE 1 (COI1) that acts as the JA receptor in vivo and, in response to JA-Ile, targets the JAZ repressors for proteolytic degradation. The co-repressors Novel Interactor of JAZ (NINJA) and TOPLESS (TPL) mediate the repressing effect of JAZ proteins (Santner and Estelle 2007). In the absence of JAs, members of the JAZ protein family repress expression of JA-responsive genes by inhibiting MYC2 activity. Upon treatment with the hormone, JAZ proteins interact with COI and are degraded by UPS, thereby allowing JA-responsive genes activation. In the absence of JAs, members of the JAZ protein family repress expression of JA-responsive genes by inhibiting MYC2 activity. Upon treatment with the hormone, JAZ proteins are degraded by the UPS, thereby allowing transcriptional activation of JA-responsive genes. This degradation depends on the direct interaction between JAZ and COI1 (Nagels Durand et al. 2016). JAZ proteins contain three conserved domains: the zinc-fingers expressed in the inflorescence meristem (ZIM) domain, a region of weak homology at the N-terminus and the C-terminal Jas domain which is most strongly conserved. The Jas domain is essential for JAZ stability because it constitutes the interaction platform between JAZ and COI1 upon hormone treatment. The Jas domain of JAZ repressors is required for both the formation of the COI1-JA-Ile-JAZ co-receptor complex and the interaction with the JID domain of MYC TFs.

3.2 Examples of Limited Proteolysis in Hormonal Regulation

3.2.1 Auxin

The plant hormone indole-3-acetic acid (IAA or auxin) regulates many developmental processes and stress responses, acting as permissive or restrictive signal depending on concentrations of active auxin or tissue/cell context. Auxin promotes the recognition of the transcriptional repressors Aux/IAAs “degron” domain II (DII) by the E3 Ub-ligase proteolytic complex Skp1–Cullin1–F-box (SCF)TIR1/AFB, leading to their degradation in the nucleus. In the apical hook, a parallel cytoplasmic pathway commences at the cell membrane and involves TMK1 (Cao et al. 2019). TMK proteins contain an intracellular kinase domain, a single transmembrane pass and an extracellular domain with two leucine-rich repeats separated by a non-LRR region. *tmk1* loss-of-function mutants have disrupted apical hook development. TMK1 shows a transient cytosolic and nuclear distribution at the concave side of the apical hook. The redistribution depends on the cleavage of TMK1 by an unknown pathway that coincides with local auxin maxima, releasing a fragment containing the intracellular kinase domain, dubbed as TMK1-C (Cao et al. 2019).

In strict contrast to the Skp1–Cullin1–F-box $(SCF)^{TIR1/AFB}$ digestive pathway, TMK1-C stabilizes IAA repressors (Cao et al. 2019). The stabilization step involves the translocation of TMK1-C from the plasma membrane to the cytosol and nucleus where it interacts with and phosphorylates IAA32/34. The two proteins lack the DII and thus are not degraded by $SCF^{TIR1/AFB}$. In the *tmk1*, IAA32/34 proteins decreased, and auxin could not induce their accumulation. However, it is unclear what determines the stability of IAA32/34 and these proteins could contain cryptic degrons or are degraded by non-UPS pathways such as exoproteases.

Another example of a limited proteolytic pathway regulating auxin is the aforementioned *Arabidopsis* ESP (see Sect. 3.1). ESP regulates auxin gradient in the root meristem by adjusting PINFORMED (PINs) proteins which give rise to directional polar auxin transport and contribute to auxin levels regulation (Liu et al. 2017, 2020; Moschou et al. 2016). This regulation is accomplished through a collaboration between ESP and microtubule-binding kinesins (Kin7.3-clade), affecting microtubule stability and delivery of PINs to polar plasma membrane domains. There, PINs transport auxin contributing to a certain distribution. Although microtubules are important in the regulation of PIN polarity, the molecular mechanism by which ESP executes the polarization of PINs is unknown.

3.2.2 Ethylene

Ethylene is a volatile plant hormone involved in many plant processes such as ripening, ageing and senescence, and is produced in all plant organs. Ethylene is perceived at the ER membrane (Chao et al. 1997). When ethylene is absent, specific ethylene receptors form complexes with the protein kinase CONSTITUTIVE TRIPLE RESPONSE-1 (CTR1) and the integral membrane protein ethylene insensitive 2 (EIN2), a process which leads to EIN2 phosphorylation by CTR1 (Hua and Meyerowitz 1998).

EIN2 is a general regulator of the ethylene signalling pathway. When ethylene is present, it acts as an inverse agonist by inhibiting its receptors. EIN2 is, then, released, dephosphorylated and cleaved releasing a fragment with two functions. On the one hand, the EIN2 C-terminal part is translocated to the nucleus, where it stabilizes the EIN3 transcription factor via the EIN2 nuclear-associated protein 1 (ENAP1), resulting in the activation of ethylene response genes (Wen et al. 2012). On the other hand, the EIN2-C is bound, occasionally, to EBF1 and EBF2 mRNAs, where this complex is associated with processing bodies in the cytoplasm, leading to degradation. Degradation of EBF1 and EBF2 releases, among others, EIN3 and results thus in more ethylene signalling. Interestingly, the protease executing EIN2 cleavage is unknown.

3.3 Peptidic Hormone-Like Molecules

Small posttranslationally modified peptides are signalling molecules involved in many aspects of plant growth and development (Stührwohldt et al. 2020). One of the posttranslational modifications that peptides undergo is proteolytic cleavage. As exemplified in Sect. 3.2, subtilisins (SBTs) are amongst the most probable candidates for diffusible peptide production. SBTs are usually produced as inactive proteins, activated upon removal of their inhibitory leading peptides through autoprocessing. Once auto-processed, they cleave substrates in the apoplast (Vartapetian et al. 2016).

The discovery of SBT substrates and their functional studies have been obscured by extensive redundancies within the large SBT family (56 members in *Arabidopsis*; (Schardon et al. 2016). A notable exception of SBTs' "redundancy rule" is SBT5.2 that modulates the density of stomata in high levels of CO2, by producing the mature peptide EPIDERMAL PATTERNING FACTOR 2 (EPF2). In the apoplast, EPF2 inhibits epidermal cells from adopting a meristemoid mother cell fate (MMC; (Engineer et al. 2014). By combining expression profiling with proteomic studies, Engineer et al. identified SBT5.2 as the potential protease cleaving EPF2. EPF2 cleavage by SBT5.2 was confirmed *in vitro* and the double loss-of-function mutant *epf2sbt5.2* had no additive phenotypes compared with the single mutants, suggesting that EPF2 and SBT5.2 act together. EPF2 overexpression in the *sbt5.2* background reduced stomata to a lesser extent than in the wild-type, indicating SBT5.2 involvement in EPF2-peptide maturation.

Peptides can mature in a stepwise manner in different cell compartments. The peptides CLEL6 and CLEL9 (also known as GOLVEN 1 and 2) control gravitropic responses of the shoot and the root by modulating auxin distribution (Stührwohldt et al. 2020). Several proteolytic processing events located in consecutive compartments of the secretory pathway are required for maturing CLEL peptides. Using an inhibitor-based approach for loss-of-function analysis, targeting protease function at the level of enzyme activity it was shown that the stepwise maturation of CLEL peptides is mediated by SBTs. Following the cleavage of the signal peptide upon entry into the ER, the CLEL6 and 9 precursors are processed at two sites by SBT6.1. Cleavage by SBT6.1 in the *cis*-Golgi allows for the continued passage of the partially processed (pre-activated) precursors through the secretory pathway and is thus a prerequisite for subsequent posttranslational modifications including tyrosine sulfation and proline hydroxylation within the Golgi, and proteolytic maturation after exiting the Golgi (Stührwohldt et al. 2020). The activation of CLEL6 and CLEL9 by SBTs in the *trans*-Golgi network or other post-Golgi compartments depends on the Nt aspartate of the mature peptides.

As mentioned earlier, extensive redundancies within SBTs (or other protease families) make them refractory to functional studies. To overcome this limitation, Schardon et al. expressed heterologous SBT inhibitors from *Phytophthora infestans*, called extracellular proteinase inhibitors (EPIs; EPI1 and EPI10), under specific promoters active during floral organ abscission. EPI expression blocked the production of the diffusible, (IDA) mature form (mIDA), which controls floral abscission in *Arabidopsis* (petals, sepals and stamens), and a similar pathway functions in root cap sloughing (Shi et al. 2018). The SBT4.12, SBT4.13 and SBT5.2, expressed during abscission, could cleave IDA *in vitro*. Although these data are insufficient to conclude how many SBTs are involved in the abscission, they provide evidence for SBTs involvement in this process.

4 Proteolysis in Leaf Development and Endoreduplication

During leaf development, the balance between cell proliferation and differentiation is highly coordinated even before the new leaf emerges and grows. Endoreplication permits multiple rounds of DNA replication without subsequent cell division (cytokinesis), leading to the successive doubling of the nuclear DNA content of the cell (Dissmeyer et al. 2009). The transition from a mitotic cell cycle into an endocycle is often associated with the switch of meristematic cells from division to expansion and differentiation. Proteolysis ensures the unidirectional progression of the cell cycle by causing irreversible changes in key factors that direct this process. The cyclin-dependent kinase inhibitor KRP2 is considered a crucial component to whether the cell will undergo endoreplication through the regulation of mitotic Cyclin-Dependent Kinase (CDK)A;1 complex (De Schutter et al. 2007). More specifically, KRP2 expression levels are regulated posttranslationally via proteolysis in a phosphorylation-dependent way and the abundance of this protein determines the inhibition of CDKA;1, thus, resulting in mitotic progression or endocycle entrance.

Previous studies showed that regulated proteolysis, especially UPS, is considered an important factor for leaf growth. Two E3 ligases, BIGBROTHER (BB) and HISTONE MONOUBIQUITINATION (HUB1, and its homolog HUB2) define cell proliferation phase length and rate in leaves by tagging with Ub the proteins that need to undergo degradation. More specifically, HUB1 can mono-ubiquitinate histone H2B *in vitro* and the corresponding knockdown mutants showing increased cell cycle duration in young leaves. That led to reduced rosette biomass, pale leaves and changes in leaf shape (Fleury et al. 2007). Regarding the BB protein, changes in expression levels lead to different organ sizes and BB abundance correlates with cell proliferation (Cattaneo and Hardtke 2017). The *Arabidopsis* Ub-activated protease DA1 limits the duration of cell proliferation during organ growth. DA1 is activated by BB and another RING E3 ligase and the DA2. The BB and DA2 are cleaved by DA1 and this cleavage leads to their destabilization (Dong et al. 2017). The cleavage of BB leads to destabilization via the N-end rule pathway-related protein PRT1. DA1 protease also cleaves the de-ubiquitinase UBP15 and the transcription factors TEOSINTE BRANCHED 1/CYCLOIDEA/PCF 15 (TCP15) and TCP22 which repress endoreduplication and promote cell proliferation. Furthermore, another example of cell cycle regulation through proteolysis during leaf development is the Anaphase-Promoting Complex/Cyclosome (APC/C) which is a Ub E3 ligase that targets mitotic cyclins for degradation by the UPS (Kondorosi et al. 2005). Eloy et al. overexpressed the subunit 10 of this complex and observed enhanced leaf size due to increased APC/C activity that led to higher cell division rates, while knockout mutant showed strong defects in female gametogenesis (Eloy et al. 2011).

Finally, another CDK activity repression pathway includes the SIAMESE(SIM) gene that encodes the founding member of the SIAMESE-RELATED family. While normally, the trichomes of *Arabidopsis* undergo three to four endoreplication cycles, in *sim* mutants this does not occur due to endocycle repression, leading them to divide mitotically (De Veylder et al. 2011). Furthermore, the SIM homolog SMR1/LOSS OF GIANT CELLS FROM ORGANS (LGO) is upregulated in developing leaves and sepals, indicating a more general role in modulation of endoreplication transition during organ emergence (Roeder et al. 2012).

5 Proteolysis in Senescence

Senescence is the last developmental phase that eventually results to death of single cells, tissues or even the whole plant. This stage is highly regulated and accompanied by an increased rate of protein degradation. It can also function as a recycling process by which the nutrients accumulated in senescing tissues are remobilized to be used for the production of new vegetative, reproductive or storage organs. While the mRNA levels of most genes decline, those of "senescence-associated genes" (SAGs) increase during senescence. As protein degradation is a vital process for successful nutrient recycling, it is not surprising that many of the upregulated genes in senescing tissues encode proteases (Bhalerao et al. 2003).

Among the many classes of plant proteases, mostly serine and cysteine-dependent proteases associate with senescence across different species (Diaz and Martinez 2013). Serine proteases are the largest protease class in plants and evidence shows that they are the dominant proteases during senescence (van der Hoorn 2008). For example, two SBTs (P1 and P2) are activated during dark and nitrogen-starvation induced senescence in wheat and execute ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) degradation in developing grains (Roberts et al. 2011).

At least three cysteine proteases are induced in the vacuole during dark-induced senescence in wheat (Martínez et al. 2007). Another cysteine protease is the SAG12, a papain-like protease. However, the physiological role of SAG12 is not known; the loss-of-function *sag12* mutant neither fails to develop senescence-associated vacuoles nor shows any morphological phenotype. The Vacuolar Processing Enzyme (VPE) cysteine protease is responsible for vacuolar protein maturation and promotes cell death during stress and development (Nakaune et al. 2005). In *Arabidopsis*, the isoforms αVPE and γVPE are upregulated in vegetative organs during senescence (Sanmartin et al. 2005). Finally, plant metacaspases are upregulated in *Arabidopsis* during senescence in leaves (MC6 and MC9) and flowers (MC3 and MC9), although their exact mechanistic function remains elusive (Breeze et al. 2011).

The class of aspartic proteases is also linked to senescence, although their role is less documented than cysteine protease. Proteomic studies in different species such as oilseed rape (*Brassica napus*) and rice showed high levels of aspartic proteases, throughout senescence (Desclos et al. 2009). The most characteristic example of aspartic protease role in plant senescence is CND41 in tobacco (Kato et al. 2004, 2005). This protease localizes in chloroplast and degrades Rubisco *in vitro* at physiological pH. Besides, silencing of CND41 resulted in Rubisco accumulation in older leaves and delayed senescence suggesting deficient remobilization machinery, while overexpression led to accelerated senescence and higher Rubisco degradation in senescent leaves.

Metalloproteases have a role in senescence but to a lesser extent. The most studied metalloprotease family in plants is the FtsH, which is a family of membrane-bound ATP-dependent proteases that contain a zinc-binding domain. Twelve genes are encoding FtsHs in the nuclear genome of *Arabidopsis,* and nine of them localize in chloroplasts while the others are found in the thylakoid membrane (Kato and Sakamoto 2010). Many FtsHs are upregulated during senescence in *Arabidopsis* (Guo et al. 2004). Furthermore, studies in *Brassica napus* senescence under nitrogen starvation led to the induction of the chloroplast-localized FtsH protease at the early stages of senescence while decreasing at the end, indicating a role in early protein degradation in chloroplasts (Desclos et al. 2009).

With regard to digestive proteolysis, in both plants and mammals, autophagy has direct effects in triggering and executing cellular senescence. As mentioned above, recycling of inefficient photosynthetic organs provides plants with nutrient supplies to progress their life cycle at the whole-plant level and sustains seed quality via efficient seed filling. More specifically, leaf senescence is a dynamic process that responds to source/sink demands of the plant regulated by stress responses, nutrients sensing and phytohormones (Buchanan-Wollaston 1997; Lim et al. 2003; Guiboileau et al. 2010). Autophagy plays an important role during senescence as defects in autophagy generally have almost normal development but are more sensitive in nitrogen and carbon starvation or progress to early senescence in low light conditions (Yoshimoto et al. 2014; Thompson et al. 2005; Chung et al. 2010). One exception is the loss-of-function *atg6* knockout mutant which shows defective pollen germination (Fujiki et al. 2007) and therefore there can be no homozygous mutants. On the other hand, in mammals, knockout of ATG genes may lead to embryo lethality while from a molecular point of view, elevated levels of autophagy activation may induce cell death, whereas decreased autophagy triggers cellular senescence (Kuma et al. 2005; Rajendran et al. 2019). Furthermore, loss-of-function *atg9* mutants in *Arabidopsis* showed early leaf chlorosis (degradation of chlorophyll) under nitrogen or carbon-starvation conditions (Zhuang et al. 2017).

6 Organellar Proteolysis and Development

6.1 Mitochondria

In mitochondria, protein turnover is regulated externally via anterograde/retrograde communications with the nucleus and internally via proteolysis and chaperones that act posttranslationally on the proteome. Mitochondrial protein turnover is controlled by ATP-dependent proteases involved in the degradation of misfolded proteins or limited proteolytic cleavage of pre-sequences, resulting in protein maturation. ATP-dependent proteases have two conserved domains, an AAA+ domain which enables the protease to bind ATP and performs as a chaperone and a proteolytic domain with a Serine-K catalytic dyad (Lee and Suzuki 2008; Rigas et al. 2009). Among others, long-filament phenotype-1 (LON1), caseinolytic protease (CLPP) and filamentous temperature-sensitive H (FTSH) are the most dominant and conserved components of the proteolytic networks in eukaryotes (van Wijk 2015).

CLPP is an energy-dependent serine-type protease that plays a role in protein quality control. The CLPP protease has an active Serine-Histidine-Aspartate catalytic triad and is present widely amongst bacterial species as well as fungal, mammalian and plant mitochondria (Bhandari et al. 2018). In plants, knockouts of the single gene for CLP protease subunit, *clpp2,* did not result in growth or development defects, nuclear transcripts were unaffected, whereas mitochondrial genes encoding oxidative phosphorylation protein complexes were abundant (Petereit et al. 2020).

LON1 is a highly conserved mitochondrial protein family in eukaryotes acting as a chaperone facilitating the proper folding of newly synthesized or imported proteins in the mitochondria and as a protease removing protein aggregates (Li et al. 2017). Mitochondrial LON1 loss impairs oxidative phosphorylation complexes and tricarboxylic acid cycle (TCA) enzymes. Phenotypically, LON depletion in *Arabidopsis* restricts root growth and leads to deleterious, developmental phenotypes (Rigas et al. 2009). When LON1 is disrupted, oxidative phosphorylation proteins are less abundant, resulting in heat shock proteins and prohibitin accumulation in the mitochondria (Solheim et al. 2012).

FtSH proteases are membrane-bound metalloproteases family abundant in eubacteria, animals and plants. So far, we know four FtSH genes in *Arabidopsis* mitochondria; FtSH3 and FtSH10 are mitochondrial AAA proteases, while FtSH4 and FtSH11 function as mitochondrial inner membrane AAA proteases (Kato and Sakamoto 2010). More specifically, FtSH4 is involved through reactive oxygen species accumulation, in phytohormone signalling and transcriptional regulation (Zhang et al. 2017b). FtSH4 substrates include the inner membrane translocase Pam18-2 and mitochondrial pyruvate transporter 4 (MPC4) (Opalińska et al. 2017). In *Arabidopsis* FtSH4 loss of function results in abnormalities of rosette leaves when grown under short-day conditions, structural alterations in mitochondria, accompanied with increased reactive oxygen species levels, carbonylated mitochondrial proteins, reduced cardiolipin contents (Gibala et al. 2009), oxidative phosphorylation subunits alterations (Heidorn-Czarna et al. 2018) and Hsp70 and prohibitins accumulation (Gibala et al. 2009). Additionally, evidence shows that FtSH4 is involved in auxin homeostasis, regulating plant growth and development (Zhang et al. 2014), as well as the regulation of autophagic-PCD and senescence through SA homeostasis (Huang et al. 2018). Moreover, FtSH4 is essential for oxidative phosphorylation subunits biogenesis and, thus, biogenesis of mitochondria during germination (Heidorn-Czarna et al. 2018). Overall, though, recent studies show that mitochondrial AAA proteases, despite their importance in translation and mitochondrial proteome surveillance, do not seem to have serious morphological modifications in plants grown under normal conditions (Kolodziejczak et al. 2018).

6.2 Chloroplasts

Nucleus-encoded proteins constitute a large part of the organellar proteome. These proteins are imported by multiprotein translocases (TOC) in chloroplast envelope membranes. Prokaryotic-type proteases control internal chloroplast protein turnover. So far, we know three procaryotic-like proteases modulating chloroplast protein turnover: FtsH, Deg and Clp. Overall, we so far know 20 chloroplast proteolytic types. Specifically, processing peptidases and energy-driven processive proteases are the major players in chloroplast proteome biogenesis, remodelling and maintenance.

6.2.1 The CHLORAD Pathway

The most well-studied example of proteolysis in chloroplasts is photosystem turnover regulation during excess light which can damage the photosynthetic mechanism. More specifically, photosystem II reversible phosphorylation and dephosphorylation lead to the E3-dependent ubiquitinilation and degradation of its D1 domain. This D1 domain has to be synthesized again *de novo* so that the photosystem II complex can perceive light and participate again in the photosynthetic pathway (Nath et al. 2013). On the other hand, targeted protein turnover at the chloroplast outer membrane is perhaps mediated by cytoplasmic proteolytic pathways. For example, the outer membrane CDC48 is an Omp85-type channel and SP2 is a cytosolic AAA+ chaperone that fulfils conductance and motor functions, respectively, in the retrotranslocation of target proteins from chloroplasts. This D1 domain has to be synthesized again *de novo* so that the photosystem II complex can perceive light and participate again in the photosynthetic pathway (Nath et al. 2013). CDC48 extricates proteins from the chloroplast membrane (they are integral membrane proteins). This process exposes outer membrane proteins to the UPS, of the substrates tagged by the chloroplast-localized E3 ligase SP1. In accordance, SP1, SP2 and CDC48 physically interact and form a complex at the chloroplast membrane. This proteolytic system is known as “chloroplast-associated protein degradation”, or CHLORAD (Ling et al. 2019).

Yet, we anticipate that many more proteins are CHLORAD components and possible links to chlorophagy (see below), a specialized type of autophagy targeting chloroplasts (van Wijk 2015; Izumi and Nakamura 2018; Ding et al. 2018; Tornkvist et al. 2019; Have et al. 2017). We should note, for example, that in mammalian cells, Ub-tagging largely acts as a trigger of autophagic removal of dysfunctional organelles (Marmor-Kollet et al. 2020). For example, during mitophagy (i.e. mitochondrial autophagy), dysfunctional mitochondria are tagged by the E3 ligase Parkin, allowing for the autophagic removal of tagged mitochondria (MacVicar et al. 2019; Jin and Youle 2012). We should note, for instance, that in mammalian cells, Ub-tagging largely acts as a trigger of autophagic removal of dysfunctional organelles (Marmor-Kollet et al. 2020).

6.2.2 The Autophagy-Rubisco Degradation Pathway

Chloroplasts have specialized proteolytic machineries for protein degradation during senescence. Whereas stromal proteins decrease during the earlier stages of leaf senescence in wheat or barley (*Hordeum vulgare*), chloroplast numbers/cell decrease later (Kubínová et al. 2013). Therefore, stromal proteins can degrade without the breakdown of the chloroplast. Immuno-electron microscopy (EM) analysis of Rubisco degradation in senescing wheat (*Triticum aestivum*) leaves revealed the presence of cytosol-localized small vesicles (~1 μm) that contained Rubisco, but not thylakoid proteins (Izumi and Nakamura 2018). While stromal proteins decrease during the earlier stages of leaf senescence in wheat or barley (*Hordeum vulgare*), chloroplast numbers/cell decrease later (Kubínová et al. 2013). Therefore, stromal proteins appear to degrade either inside or outside the chloroplast without the breakdown of the entire chloroplast. These vesicles are frequently surrounded by autophagosome-like double membranes and were originally referred to as Rubisco-containing bodies (RCBs). In *Arabidopsis* and rice leaves expressing stroma-targeted green fluorescent protein (GFP) or GFP-labeled Rubisco showed that RCBs are not produced in the *atg5* or *atg7* mutants and that RCBs co-localized with the autophagosomal marker, GFP-ATG8. These observations reveal that RCBs are a type of autophagic body that delivers a portion of the stromal proteins into the vacuole (Otegui 2017).

ESCRT is part of an evolutionarily conserved system responsible for endosomal membrane remodelling. In *Arabidopsis*, the endosomal sorting complex required for transport (ESCRT)-III paralogs charged multivesicular body protein 1A (CHMP1A) and CHMP1B and delivered RCBs to the vacuole (Spitzer et al. 2015). In loss-of-function *chmp1a chmp1b* mutants, RCBs accumulated in the cytoplasm; therefore, CHMP1 proteins sort RCBs. How a portion of the stroma is separated as RCBs, and how RCBs are then recruited for autophagic transport remains unclear.

The RCB pathway is particularly active in sugar-starved, excised *Arabidopsis* leaves in darkness or the presence of photosynthesis inhibitors. The starchless mutants, phosphoglucomutase (*pgm*) and ADP-glucose pyrophosphorylase1 (*adg1*), lacking starch, showed enhanced RCBs production (Izumi et al. 2010). Moreover, starchless and *atg* double mutants have reduced growth and enhanced senescence compared to the respective single mutants. These findings indicate that the RCB pathway mediates nitrogen remobilization from older leaves that cannot acquire sufficient light due to the shading of developing leaves by upper tissues. Analyses of Arabidopsis and maize (*Zea mays*) plants with *atg* mutations showed that autophagy contributes to nitrogen remobilization from vegetative tissues to reproductive tissues and seeds (Li et al. 2015). However, such a role for autophagy in rice plants was not evaluated, because autophagy-deficient rice plants exhibit male sterility due to impaired pollen maturation (Hanamata et al. 2019; Sera et al. 2019).

Some isolated vacuoles from the darkened leaves of wild-type plants contained chloroplasts that exhibited chlorophyll autofluorescence signals. These findings suggest that shrunken chloroplasts, which are produced through the active separation of their components in the RCB pathway, become the targets of autophagic transport as entire organelles, a process known as chlorophagy (Yamauchi et al. 2019). ATG8-interacting protein 1 (ATI1) and ATI2 were identified in a yeast two-hybrid screen as candidates that interact with the *Arabidopsis* ATG8 isoform, ATG8f (Wu et al. 2021; Michaeli et al. 2014). These proteins associate with plastids (in addition to the ER) as small vesicles of approximately 1 μm in diameter, which are referred to as ATI bodies. These delivery cargos differ from those of the RCBs that specifically contain a portion of stroma; however, the vacuolar transport of plastid-associated ATI bodies is an autophagy-dependent process, as ATI was not produced in *atg5* mutants (Michaeli et al. 2014). Therefore, ATI bodies represent a distinct form of autophagy vesicles that transport some stroma, thylakoid and envelope components into the vacuole. Plastid-associated ATI bodies are also observed inside the chloroplast, and ATI1 interacts with some thylakoid proteins *in vivo*. It is thus conceivable that plastid-associated ATI bodies form in chloroplasts and are then delivered into the vacuole via autophagosome-mediated transport, although how such bodies are evacuated from chloroplasts remains unclear.

6.2.3 The SAV Pathway

Senescing leaves of *Arabidopsis*, soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*), and perhaps other plants, show small, lytic senescence-associated vacuoles (SAVs). SAVs are stained by the R-6502 dye, emitting strong fluorescence upon the hydrolytic activity of cysteine proteases (Martínez et al. 2008). SAVs form in the peripheral cytoplasmic region of mesophyll cells and are much smaller than the central vacuole, but with much greater lytic activity. SAVs contain SAG12, stromal proteins such as Rubisco and glutamine synthetase, but not thylakoid proteins such as LHCII and the photosystem II reaction centre D1 (Martínez et al. 2008). As *atg7* mutants had SAVs, they may be an autophagy-independent, extra-chloroplastic route for stromal protein degradation in senescing leaves. The trafficking pathway of stromal proteins into the SAVs, as well as their composition in proteases, remains uncertain.

6.2.4 The CV Pathway

Another type of organelle related to protein degradation is chloroplast vesiculation (CV), which depends on a protein produced by the CV gene. In *Arabidopsis*, CV-GFP expression under the dexamethasone-inducible promoter caused the formation of a type of chloroplast-derived vesicle showing a strong signal referred to as

CV-containing vesicles (CCV) (Kamranfar et al. 2018). CCVs are around 1 μm and contain stroma, envelope and thylakoid proteins, as shown by immunoblot analysis of some chloroplast proteins, co-immunoprecipitation assays and detection of fluorescently tagged microscopy of stroma markers. CCVs do not associate with GFP-ATG8a and SAVs, and the *atg5* mutation does not affect CCVs. Thus, CCVs are part of a vacuolar degradation process for chloroplasts that is independent of autophagy and SAVs.

6.2.5 "N-end rule" Pathway in Chloroplasts

Similar pathways to N-end rule may also function in organelles. Nuclear-encoded proteins comprise $>$ 95% of the chloroplast proteome and are targeted to the plastid by an Nt-chloroplast transit peptide (cTP). Upon delivery to the chloroplast, the cTP is removed by the stromal processing peptidase (SPP) to expose new Nt-amino acids, which can then be further modulated by one of at least seven aminopeptidases (van Wijk 2015). SPP cleaves at a range of different sites, and single or multiple positions; this enzymatic promiscuity coupled with subsequent amino-peptidase activity may ensure the removal of unfavourable (potentially destabilizing) Nt-residues (Rowland et al. 2015). Furthermore, plastid-encoded proteins initiate with Nt-fMet and undergo cotranslational de-formylation followed by Nt-Met excision, both essential for normal plastid development (Giglione et al. 2014). Interestingly Met-retention on chloroplast proteins associates with protein instability (Giglione et al. 2003), whilst fMet can act as a destabilizing residue in bacteria, and possibly also chloroplasts (Piatkov et al. 2015). Cotranslational and posttranslational Nt-acetylation also occurs on chloroplastic proteins, which appears to enhance protein stability (Bienvenut et al. 2011); a nuclear-encoded chloroplast-targeted NAT probably catalyses this modification (Disch et al. 2006).

6.3 Peroxisomes

Peroxisomes are organelles, abundant in all eukaryotes and essential for development and stress responses. Some of their important cellular functions are fatty acid β-oxidation, photorespiration, ureide and polyamine metabolism, biosynthesis of plant hormones and reactive oxygen/nitrogen species production (Moschou et al. 2008; van Wijk 2015). Peroxisomes metabolize the auxin indole-3-butyric acid (IBA) into the active auxin indole-3-acetic acid (IAA) (reviewed in Strader and Bartel (2011)). Mutants with dysfunctional peroxisomes often display impaired IBA-to-IAA conversion (Strader and Bartel 2008) and dampened IBA responsiveness (Zolman et al. 2008).

Arabidopsis peroxisomes contain several proteases, e.g. LON2 (Lon protease 2), DEG15 (Degradation of periplasmic protein 15), SCPL20 (Serine carboxypeptidase-like protein 20), RDL1 (Response to drought21A-like 1) and PXM16 (Peroxisomal

M16 metalloprotease) (Pan and Hu 2018). LON2 facilitates sustained matrix protein import in mature peroxisomes and the degradation of matrix proteins during peroxisome remodelling (Adebesin et al. 2018). LON2 facilitates sustained matrix protein import in mature peroxisomes and the degradation of matrix proteins during peroxisome remodelling (Adebesin et al. 2018). In watermelon, DEG15 can be a processing peptidase as a dimer to cleave PTS2 from PTS2-containing proteins or a general protease in its monomeric form (Helm et al. 2007). SCPL20 is involved in β-oxidation and plant pathogen response (Quan et al. 2010, 2013), and RDL1 plays a role in β-oxidation, seed viability and stress response (Quan et al. 2013). The exact role of RDL1, SCLP20 and PXM16 remains to be elucidated.

Plant peroxisomal proteome varies to some extent in different developmental stages and tissue types. During germination, enzymes in peroxisomes catalyse β-oxidation and the glyoxylate cycle, thereby allowing lipids stored in seeds to be used as energy before photosynthetic machinery commences. As phototrophy is established several days after germination, peroxisomes are remodelled to carry out the glycolate pathway required for photorespiration. This remodelling most likely involves the simultaneous action of several processes, such as LON2-mediated degradation of the glyoxylate cycle enzymes and a specialized autophagy type called “pexophagy”. LON2 dysfunction results in glyoxylate cycle enzymes stabilization (Farmer et al. 2013; Goto-Yamada et al. 2014). Besides its protease activity, LON2 also has chaperone activity that suppresses pexophagy and peroxisome remodelling (Goto et al. 2014). Interestingly, the protease activity of LON2 seems to interfere with its chaperone-dependent inhibition of pexophagy and thus helps to control the protection of peroxisomes from autophagy. Remodelling of the peroxisomal proteome is also attributed to light- and sugar-dependent transcriptional changes, and possibly other proteases, the UPS and proteins involved in peroxisome biogenesis (Goto et al. 2015).

All examined *Arabidopsis lon2* mutant alleles are resistant to IBA-induced lateral root formation but respond normally to auxins not requiring β-oxidation (Lingard and Bartel 2009; Burkhart et al. 2012), suggesting reduced IBA-to-IAA conversion. Additionally, *lon2* mutants display age-dependent defects in PTS2 processing that are accompanied by defects in peroxisomal matrix protein import, indicating that LON2 is necessary for the sustained import of matrix proteins. Despite these defects in peroxisome physiology, disrupting *Arabidopsis* LON2 does not appear to result in matrix protein stabilization.

A second peroxisomal protease with a role in *Arabidopsis* matrix protein degradation is DEG15 (Schuhmann et al. 2012). The family of Deg proteases (for degradation of periplasmic proteins), also known as HtrA proteases (for high-temperature requirement A), are one important group of these proteolytic enzymes (Schuhmann et al. 2012). Degs are ATP-independent serine endopeptidases found in all domains of life, including Bacteria, Archaea and Eukarya. DEG15 processes PTS2 proteins into their mature forms by removing the Nt PTS2-containing (Helm et al. 2007; Schuhmann et al. 2008), but DEG15 is not required for isocitrate lyase or malate synthase degradation (Lingard and Bartel 2009). Similarly, the peroxisomal M16 metalloprotease PXM16 is not required for degradation of glyoxylate cycle enzymes; *pxm16* and *lon2 pxm16* mutants efficiently degrade isocitrate lyase (Lingard and Bartel 2009).

Pexophagy is involved in peroxisomal quality control and removes damaged catalase aggregates and clustered peroxisomes (Kim et al. 2013; Shibata et al. 2013; Yoshimoto et al. 2014; Hackenberg et al. 2013). The plant pexophagy receptor for ATG8 remains elusive. A bioinformatics approach named hfAIM (high fidelity ATG8 interacting motif) identified 9 peroxisomal PEX proteins in *Arabidopsis* that contain putative hfAIM, among which PEX6 and PEX10 were further verified by bimolecular fluorescence complementation (Xie et al. 2016). Yeast two-hybrid screen identified PEX10 as an ATG8-interacting protein, suggesting that PEX10 is a promising candidate for a receptor in pexophagy (Marshall et al. 2019). A recent study showed that autophagy mediates glucose-promoted peroxisomal degradation in roots and that ATG8 physically interacts with a peptide of PXA1 (Peroxisomal ABC transporter 1)/CTS (Comatose)/PED3 (Peroxisome defective 3) (Huang et al. 2019), making PXA1 another possible receptor for pexophagy. Whether full-length PXA1 interacts with ATG8 in planta has not been shown. More rigorous studies will determine whether any of these ATG8-interacting proteins function as true pexophagy receptors.

6.4 Endoplasmic Reticulum

The ER environment is very sensitive and can result in cytotoxic conditions known as ER stress, which can compromise the balance between protein folding and synthesis. ER stress is under continuous surveillance and proteolytic pathways execute the removal of misfolded proteins. Processes including the unfolded protein response (UPR), ER-associated degradation (ERAD) and autophagy play important roles in restoring ER homeostasis. For excellent reviews on ERAD and UPR, we refer readers to Chen et al. (2020); Bao and Howell (2017).

In plants, we know two ER stress sensor branches: the inositol requiring enzyme 1 (IRE1) and the activating TF 6 (ATF6). IRE1 pathway orchestrates UPR and is the most evolutionary conserved branch of the UPR. The ATF6 (basic leucine zipper protein (bZIP)) are ER transmembrane proteins with a cytosolic Nt containing bZIP transcription factor domain and an ER C-terminal domain containing ER-retention signals (Vitale et al. 2019; Iwata et al. 2008). When cells encounter ER stress, the ATF6 complex translocates to the Golgi where it is activated via regulated intramembrane proteolysis (RIP) (Brown et al. 2000). RIP releases ATF6. This biological process is mediated via proteases and results in the release of the now active bZIPTF domain of the ATF6 complex to the cytosol which is then translocated to the nucleus, regulating ER stress responses (Pastor-Cantizano et al. 2020).

IRE1 modulates UPR in plants and is a single-pass transmembrane protein that has a lumenal domain and cytoplasmic domain, conferring different functions at both ER membrane sides. IRE1 contains both RNase and protein kinase domains, and the RNase activity, but not the protein kinase activity, of IRE1 is critical for ER stress-induced autophagy. In addition to clearing unfolded proteins under stress, ERAD also functions in the turnover of normal proteins to regulate cell development, such as HMG COENZYME A REDUCTASE (HMGCR) in mammals (Menzies et al. 2018). In development, ERAD functions seem conserved from mammals to plants. The mouse homolog of Ubc6p, UBE2J1, is required for spermiogenesis, evidenced by UBE2J1$^{-/-}$ mice sterility (Koenig et al. 2014). Interestingly, in tomato (*Solanum lycopersicum*) SlUBC32 loss of function homolog of UBE2J1 results in the late-ripening of tomato fruit (Wang et al. 2014). Besides, in rice OsDER1 endosperm-specific knockdown induced UPR and resulted in abnormal phenotypes of seed size and starch granules. OsDER1 associates with OsHRD1, OsHRD3 and OsCDC48, suggesting that the HRD1-mediated ERAD pathway controls rice seed development (Qian et al. 2018).

ERAD may regulate auxin signalling. ER stress triggers the accumulation of AUX/IAA transcriptional repressors by downregulating auxin receptors and transporters in *Arabidopsis* (Chen et al. 2020). More research will determine ERAD involvement in other phytohormone-dependent growth regulation (Zhao et al. 2014). The same ERAD component, CER9/DOA10A/SUD1 (ECERIFERUM 9/DOA10A/SUPPRESSOR OF DRY2 DEFECTS1) that functions in both drought response and seedling development mediated by ABA signalling suggests that ERAD is essential in mediating the balance between plant growth and stress adaptation.

Furthermore, although selective degradation of ER by autophagy termed "ER-phagy" may function in plants, it is yet unclear whether it has direct roles in development. For more information on this topic, we refer readers to Bao and Bassham (2020). C53 protein is an ER-phagy receptor shared by plants and mammals. C53 harbours a unique shuffled ATG8 interacting motif (AIM) with ATG8 and is recruited to autophagosomes upon ER stress. C53 senses ER-associated stalled ribosomes and forms a tripartite receptor complex with the UFMylation ligase components UFL and DDRGK1, resulting in the degradation of nascent ER proteins. The corresponding *c53* mutants are therefore more ER stress-sensitive (Stephani et al. 2020). C53 thus provides an alternative quality control pathway for the maintenance of ER homeostasis.

7 Closing Remarks

The previous proteolytic pathways highlight proteases' importance in the regulation of aspects of plant development (Fig. 2). The scarcity of information on relevant protease substrates is the major bottleneck in fully elucidating proteolytic pathways affecting development. Although proteolytic pathways have been traditionally linked to intrinsically destructive processes, we believe that this might be an oversimplification of a more profound and general role of such pathways throughout the life span of a cell. The endeavour of "de-orphanizing" proteases, finding their substrates may include high-resolution expression analyses and proteomics (e.g. as was done for the stomatal regulator SBT5.2 (Engineer et al. 2014)). Many proteases,

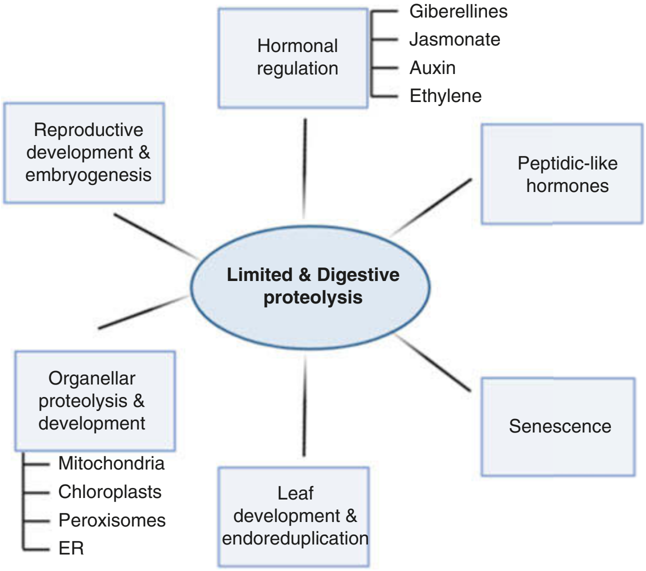

Fig. 2 Schematic representation of the most important developmental procedures, in which limited and digestive plant proteolysis are involved and described in brief in this chapter. Figure was created using BioRender (https://biorender.com)

however, are posttranslationally regulated, and therefore quantitative proteomics may not reveal their activity maxima. This problem can be solved by activity profiling methods with specific probes that can reveal the spatiotemporal protease activity (Pružinská et al. 2017). Moreover, expressing heterologous protease inhibitors from pathogens through careful selection of promoters may prove an elegant strategy to overcome genetic redundancies (e.g. SBTs in floral organ abscission; (Schardon et al. 2016)). Methods, such as positional proteomics, can be used to discover *in vivo* protease substrates and associated products of limited or digestive proteolysis. For example, the high-throughput proteomic approach dubbed COMBINED FRACTIONAL DIAGONAL CHROMATOGRAPHY (COFRADIC) revealed *in vivo* substrates of METACASPASE 9 (Wrzaczek et al. 2015; Tsiatsiani et al. 2011, 2012, 2013; Tsiatsiani and Heck 2015). Furthermore, information on protease localization is of paramount importance in inferring possible functions of proteases and relevance of the identified interactome, allowing researchers also to focus positional proteomics in a subset of the proteome, increasing the discovery rate of functionally relevant substrates. Such frameworks will streamline future efforts to decipher proteolytic pathways in an unthinkable depth, creating opportunities for practical applications.

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Experimental Evidence for Fruit Memory and Its Applications to Post-harvest Physiology and Technology: An Overview

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Contents

Abstract Memory is a phenomenon that allows organisms, including plants, to store and recall information from previous events and then change their responses to future stressful conditions. The mechanisms of memory in plants seem to have (epi)genetic, metabolic, and also electrical bases. Fruits, like other plant modules, are subject to different stimuli from the environment and the plant that bears them. They are expected to have the ability to retain and use information and to be able to send signals to the rest of the plant. In the post-harvest, priming and acclimation behind several storage techniques are closely related to memory. Here, we revisited the literature addressing memory-related traits in fruits and we propose it as

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experimental evidence for fruit memory. Additionally, we offer an experimental design to test memory in fruits. Several studies related to fruit acclimation and resistance have demonstrated memory effects. Pre- and post-harvest treatments and stresses, of biotic and abiotic origin, can induce long- and short-term memory in fruits. An example is the use of pre-treatments with low temperatures to acclimate fruits to freezing temperatures and reduce cold injuries. A new perspective in this study, such as attributing memory to fruit, may help to find new solutions to improve post-harvest shelf life and quality, as well as a better understanding of the mechanisms behind the observed responses. Still, one of the major problems is the lack of experiments designed specifically to test the hypothesis of fruit memory. To complete our contribution in this work, we propose an experimental model to test fruit memory involving pre- and post-harvest stimuli.

Keywords Acclimation, Cross-tolerance, Electrical memory, Epigenetic, Metabolism, Post-harvest storage, Priming

1 Introduction

A common characteristic of living beings is the ability to retain information about past experiences to deal with new challenges in the future (Witzany 2018). This ability is called “memory” and happens in many different levels, from the well-known immunological memory of animals and plants to the conscious, declarative memories of humans (Zinkernagel et al. 1996; Manns and Eichenbaum 2006; Pradeu and Du Pasquier 2018). However, memories do not need to be conscious and normally refer to metabolic or epigenetic adjustments of an organism after some experience. These adjustments modify the future behavior of the organisms, either in positive or negative ways when similar situations are faced again. Therefore, it is not a metaphor to attribute memory to plants (Thellier and Lüttge 2013; Witzany 2018; Galviz et al. 2020).

For instance, after some biotic stress caused by pathogens, plants can accumulate signaling proteins, such as transcription factors, which remain inactive within the cells as a memory element of the past event. If the plant re-encounters the same pathogens, these proteins could be rapidly activated by mitogen-activated protein kinases (MAPK), leading to a prompt and more effective metabolic response to the pathogen, compared with the first infection (Conrath et al. 2006; Gális et al. 2009). Another form of memory in plants is related to epigenetic changes in the chromatin, influencing a plethora of behaviors such as resistance to heat or other abiotic stresses and the regulation of vernalization (Bruce et al. 2007; Song and Ryu 2013; Crisp et al. 2016; Yang et al. 2017; Friedrich et al. 2018; He and Li 2018).

Besides, since plants can use the effects of past experiences to enhance their acclimation capabilities during lifetime (and also, transmit it epigenetically, see Quadrana and Colot 2016; Pecinka et al. 2020), one could argue that plants have a rudimentary form of learning, which is the use of memory to enhance their performance when facing situations similar to those that created that memory. Souza et al. (2018) stated that learning in plants consists of phenotypic changes based on memory that improves plant responses to a stimulus experienced in a certain moment in the past. Priming and acclimation to biotic and abiotic factors are closely related to memory. While "priming" refers to the stimuli that trigger a memory, acclimation refers to the final adaptive response to new environmental conditions supported by that triggered memory (Galviz et al. 2020). Regarding the mechanisms by which memory occurs in plants, genetic (e.g., Turgut-Kara et al. 2020), metabolic (e.g., Auler et al. 2021), and even electrical processes are involved (Volkov and Chua 2021).

The field of plant memory is rapidly expanding, and many new technologies and agricultural practices have been developed from this new paradigm (e.g., Peng and Zhang 2009; Ali et al. 2013; Song and Ryu 2018; Ribeiro and Torres 2018; Kulak 2020). It is conceivable that such development has an impact on fruit production. After all, fruits are modified leaves and stems that are part of plants until the moment they are harvested. Therefore, could they have memory too? Could studies on fruit memory be of use to the food sciences? In this work, we propose fruit as capable of storing and using memory and we discuss the potential applications of this knowledge in the post-harvest technology.

Unfortunately, until now, the discussion on plant memory has barely reached the field of food science and post-harvest technology, let alone the idea of memory in fruits. As far as we know, there is only a handful of articles assuming memory in fruits (e.g., Mattoo et al. 2007; Mattoo and Handa 2008), although a good number of studies suggest improved fruit responses depending on the previous stimuli. When attributing memory to fruits, we assume a new perspective on the phenomena that occur in fruit and that could lead to new solutions to problems that have not yet been solved, like several post-harvest storage disorders and fruit quality improvement. Considering this background, we revisited the literature that presents memory-related traits in fruits and discusses experimental evidence for fruit memory.

In the following sections, we started with a general description of fruit biology; in Sect. 2, we introduced more in-depth the concept of memory and its respective mechanisms, showing to what extent memory can be attributed to fruit; in Sect. 3, we bring experimental evidence for fruit memory. Finally, in Sect. 4 we discuss the likely impacts of regarding memory as an intrinsic capacity of fruit.

2 Fruit as Living Part of Plants

The title of this section may be obvious, but it intends to draw attention to the overlooked fact that fruits are simply seen as an edible object. However, they are crucially important modules of a plant and, as such, to a limited extent they can perceive the environment and act depending on what is sensed. As modules, they are semiautonomous biological units with more internal than external integration

(Bolker 2000; Hütt 2019; Wegner 2019), and from a certain point in their development, they can live many days and even months separated from their mother-plants.

In most angiosperms, fruits play an important role in protecting seeds. Furthermore, they are essential to attract seed dispersers (Bobrov and Romanov 2019). The predominance of angiosperms in the plant kingdom seems to indicate that the fruit's role in seed protection and dispersion has been properly accomplished (Forlani et al. 2019). Fruits remain alive when detached from plants, and some even manage to complete the ripening process (Farcuh et al. 2019). These characteristics are very important to determine when the fruits should be harvested and the strategies for post-harvest storage.

Interestingly, events that occur during the pre-harvest period can influence various parameters (Perin et al. 2019) and improve fruit response when subjected to stress factors during the post-harvest (Reissig et al. 2020). It is already established and demonstrated in the scientific literature that pre-harvest treatments and stress agents influence fruit responses after harvesting (Pedreschi and Lurie 2015; González-Chavira et al. 2018). In fact, this response can persist through the entire fruit cycle, from pre- to post-harvest. Also, fruits acclimate to recurrent stresses in the post-harvest storage (Zhang et al. 2017) and can acclimate and acquire cross-tolerance to different stress factors (Salazar-Salas et al. 2017).

Generally, fruits are the fertilized and developed ovaries of a plant, frequently united with other parts of the flower or inflorescence (Bobrov and Romanov 2019). They form a functional unity for the dispersion of seeds, which is semiautonomous from the mother-plant (especially in later stages of fruit ripening). Therefore, fruits can be considered modules of a plant (see Lüttge 2019). Except for parthenocarpic fruits, which develop in the absence of oocyte fertilization (e.g., banana), fruit development begins with the union of female and male gametes, triggering a series of modifications in the ovary tegument and surrounding tissues that will undergo physical and biochemical modifications until the ripe fruit (Bobrov and Romanov 2019). However, despite all those developmental changes, the fruit originates from pre-existing structures of the flower (e.g., carpels, pedicel, flower receptacle), which are always connected with other plant parts through vascular bundles and plasmodesmata (Bobrov and Romanov 2019).

Since all flower components are modified leaves or stems, the fruits harbor all the structures required for within-plant communication through hormones, peptides, electrical and hydraulic signals, and even volatile organic compounds such as ethylene (e.g., Barry and Giovannoni 2007; Lovisolo et al. 2010; Bisson et al. 2016; Hocking et al. 2016). Consequently, they are "equipped" for communication with other fruits and the plant itself. Accordingly, some studies have demonstrated plant-to-fruit communication through a complex, multifactorial network of signaling (Lovisolo et al. 2010), as well as fruit-to-plant signaling through electrical signals (Eschrich et al. 1988; Reissig et al. 2021).

As modular organisms, the plants' abilities to deal with the environment are distributed through the modules, and the resulting plant behavior is the emergence of the behavior of all individual modules plus the interactions among themselves (de Kroon et al. 2005, 2009; Lüttge 2021). This means that each plant module has autonomy to deal with the endogenous and environmental stimuli/information that it is receiving at a given moment. The processing and use of this information require a set of basic mechanisms such as memory, at least short-term memory, and communication (Lyon 2006; Lyon et al. 2021). There are reasons to suggest that fruits, as plant modules structured from stem and leaves, present the ability to retain and use information and communicate with the plant and other fruits. It is important because fruits are interacting with the environment, which is fickle and unpredictable, and therefore memory, at least to a minimum extent, is needed for dealing with it (Galviz et al. 2020).

As in plants, chemicals and environmental stimuli can elicit responses in fruits (Filippou et al. 2012; Pétriacq et al. 2018). Some sources of stress, such as the use of modified atmospheres and low temperatures (Pedreschi and Lurie 2015), are widely used in post-harvest to increase the quality and shelf life. The practice of using pre-treatments to prepare the fruit for subsequent stress is common in post-harvest storage (Baier et al. 2018). Many of the techniques used are based on knowledge about priming and acclimation, which are closely related to memory.

3 How to Observe Fruit Memory?

Some authors have referred to priming as the capacity to sensitize the plant immune system for a better expression of induced defense mechanisms (Pétriacq et al. 2018). Concisely, priming occurs when previous responses can affect the subsequent ones, through modifications in their metabolism, changing growth and/or morphogenesis (Galviz et al. 2020). According to Pétriacq et al. (2018), priming agents such as ß-aminobutyric acid (BABA) and R-β-homoserine (RBH) trigger induced resistance against different diseases. When BABA is applied in tomato plants (pre-harvest), the tomato fruits (post-harvest) showed reduced disease incidence to *Botrytis cinerea* (Wilkinson et al. 2018*)*. Regarding acclimation, it is related to the process of acquiring improved performance under recurrent or crossed non-lethal abiotic stimuli. Cold acclimation, for example, is a subject of interest in studies related to fruit quality, due to its importance during post-harvest storage (Salazar-Salas et al. 2017; Tanou et al. 2017; Wang et al. 2017, 2018). Pre-conditioning treatment in peaches (48 h at 20°C) before low-temperature storage (0°C) markedly altered cold responses, resulting in cold acclimation in terms of cold injury expression signatures (Tanou et al. 2017).

In an overview about induced resistance and priming to cope with fruit decay, Pétriacq et al. (2018) associated priming with memory. Because there is primed state and information retention, priming is closely related to memory and its effect, both in the short and long term. Pre-harvest stimuli can lead the fruit to show an improved response to a new stimulus during post-harvest. However, this does not tell us anything about the mechanisms behind memory, showing only its effects. Since memory is not just an effect and its elicitation, it is necessary to know how it occurs and how it is processed. As it is for animals, some memories may not be beneficial.

Induced resistance and priming have also been related to undesirable effects in fruit quality like the development of susceptible phenotypes because of cross-talks in the activation of defense mechanisms (Pétriacq et al. 2018).

Currently, there is no widely accepted model to explain all aspects of plant memory. Some proposals have been made and supported by Baluska et al. (2008), Thellier and Lüttge (2013), and Demongeot et al. (2019) and there is available literature describing biological changes associated with memory at different levels of plant organization, through different analyses and techniques. In the case of memory effect, i.e., the response observed in a previously primed plant subjected to a new disturbance when compared to a non-primed plant, there are also many studies using several approaches (Virlouvet and Fromm 2015; Leuendorf et al. 2020; Srivastava et al. 2021). Overall and considering the reported data, the establishment of relationships between the specific type of elicitor and the observed responses is a hard task and the responses seem to be grouped according to the nature of the stress (biotic or abiotic). However, it is more general and does not mean that the responses are identical for each kind of stress. Through metabolomic analysis, Luna et al. (2020) observed that ß-aminobutyric acid (BABA) resulted in a durable induced resistance in tomato fruit against *Botrytis cinerea*, *Phytophthora infestans,* and *Pseudomonas syringae* without promoting BABA-induced priming common fingerprint. The metabolomic responses observed seemed to be specific to each type of infection.

We created a general scheme to represent the possible memory mechanisms in fruits that we are describing in the following subsections, which can be epigenetic, metabolic, and even electrical (Fig. 1).

3.1 Epigenetic Memory

Among the mechanisms that support memory in plants, the epigenetic ones have gained wide attention (Fig. 1a) and we shall find intimate relations to sexual reproduction and hence fruits. In short, epigenetics consists of modifications on chromatin organization, during meiosis or mitosis, which alter gene expression but not their base sequence, and the epigenome is the collection of these biochemical modifications on DNA sequences or proteins that regulates the genome (Bernstein et al. 2007; Turgut-Kara et al. 2020; Trontin et al. 2021). Three main types of interconnected mechanisms are responsible for epigenetic regulation: DNA methylation, histone modifications, and noncoding RNAs (Thellier and Lüttge 2013; Turgut-Kara et al. 2020). Several studies have demonstrated that stress-induced environmental memory is mediated by epigenetic mechanisms, and there is a relation between priming and epigenetic memory. Changes in plants epigenome are quite stable, lasting hours to days (e.g., somatic memory) and can be transmitted to the next generation (e.g., transgenerational memory) (Lämke and Bäurle 2017; Alonso et al. 2019; Ashapkin et al. 2020; Turgut-Kara et al. 2020; Oberkofler et al. 2021).

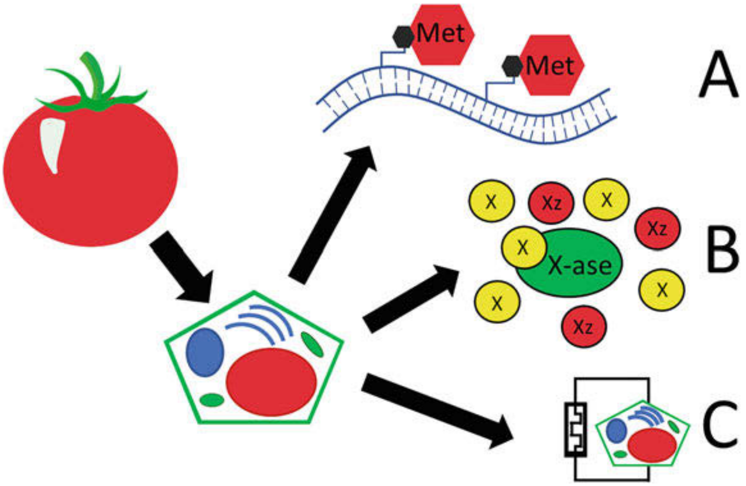

Fig. 1 Examples of possible memory mechanisms in fruit. All of them could occur simultaneously. At the cellular level, (**a**) epigenetic modifications of the chromatin could encode memory by activating or deactivating genes related to the response to the stimulus experienced, leading to a more effective response once the fruit receives a similar stimulus in the future. (Met = methylation of DNA). (**b**) Metabolic memory through the accumulation of compounds (in this case, a fictional X compound) and/or reinforcement of metabolic pathways. Once the stimulus is applied again, the accumulated compound leads to a prompter activity of the enzymes related to this pathway. In the example, the fictional "X-ase" that catalyzes X in Xz. (**c**) Electrical memory through memristor (resistor with memory), a hypothetical component of electrical circuits in plants that are dependent on the history and state of the system. Voltage-gated ion channels, proposed as memristors in plants, can regulate the generation and transduction of electrical signals along plasmodesmata and phloem

The literature has demonstrated that epigenetic features play an important role in plant tolerance/resistance mechanisms to abiotic/biotic stresses and priming (Yakovlev and Fossdal 2017; Alonso et al. 2019; Perrone and Martinelli 2020; Akhter et al. 2021; Miryeganeh 2021). Several specific epigenetic mechanisms have been already associated with priming, some examples are modulation of NPR1/NIM1, miR156 expression, H3 histone modifications (H3K4me3), DNA methylation, methyl jasmonate (MeJa) promotion of histone modifications, and DNA hypomethylation (Reyes et al. 2002; Holoch and Moazed 2015; Perrone and Martinelli 2020). Previous exposure to heat, salinity, and cold stress improved Arabidopsis resistance to *Pseudomonas syringae*. The mechanisms were epigenetically studied, and a correlation was found with the hyper-induction of pattern-triggered immunity marker genes (WRKY53, FRK1, and NHL10) as well as with increased accumulation of H3K14ac, H3K4me2, and H3K4me3, and the requirement of the histone acetyltransferase HAC1 (Pastor et al. 2013; Perrone and Martinelli 2020; Akhter et al. 2021; Miryeganeh 2021; Mladenov et al. 2021).

The epigenetic mechanisms for salinity stress memory have already been studied as well. In an experiment with Arabidopsis, plants that were primed by mild salt stress showed less salt uptake and higher drought tolerance compared with control plants. One of the findings was higher methylation levels in the primed plants for H3K4me2 and H3K4me3. Also, salt stress promotes DNA methylation in key stress-related genes such as WRKYs. To the authors, information was stored in long-term somatic memory in the shape of different chromatin statuses (Sani et al. 2013; Miryeganeh 2021). In a recent review, Perrone and Martinelli (2020) listed some of the main epigenetic mechanisms involved in plant responses to biotic stress: DNA methylation changes by herbivory attack; DNA hypomethylation at specific pericentromeric regions affected by priming and biotic stress resistance to fungal infections; regulation of the epigenetic expression of NPR1/NIM1 genes – H3 histone modifications affect stress memory in response to bacterial stress (Alonso et al. 2019).

Epigenetic memory is also pivotal for large and long-lived forest species. The stored environmental information in the seeds, like temperature conditions during zygotic embryogenesis, is crucial to the establishment of the new tree. It is also important in somatic embryogenesis (Yakovlev and Fossdal 2017; Trontin et al. 2021). Yakovlev and Fossdal (2017), using NGS sequencing and computational in silico methods, observed the influence of temperature in the epigenetic memory during somatic embryogenesis of Norway spruce. They focused on microRNAs (miRNAs) and other small noncoding RNAs (sRNAs), that play an essential role in the regulation of plant gene expression, and the results found suggested roles for novel and conserved miRNAs in the formation of epigenetic memory. Probably fine-tuning of the miRNA production participates in both developmental regulation and epigenetic memory formation. Despite the growing body of evidence, it is difficult to access the role of epigenetic processes in trees in the field. Le Gac et al. (2018) designed outdoors and greenhouse experiments to analyze the involvement of epigenetic mechanisms in the winter-dormant shoot apical meristem of *Populus* × *euramericana* clones in memory of the growing conditions faced during the vegetative period. They evaluated the global DNA methylation and analyzed the differentially and common methylated regions among treatments. The results showed that winter-dormant shoot apical meristem displays hyper- and hypomethylated regions in response to environmental variations from the preceding vegetative period. The authors suggest that DNA methylation changes during the vegetative stage are kept and correspond to stable epigenetic modifications, ergo, epigenetic memory.

Regarding reproductive structures, epigenetic mechanisms are fundamental to regulate several processes, as well as to guarantee plasticity to environmental fluctuations. A classic example of an epigenetic-related memory process associated with flowering is vernalization, i.e., the acquired ability to flowering after prolonged exposure to cold (Song et al. 2012). In vernalization-sensitive species, in addition to the floral repressor *FLOWERING LOCUS C* (FLC), histone modifications at this FLC *locus* occur when the length of winter daytime is perceived by the temperature below a threshold (Song et al. 2012; Gady et al. 2017). Interestingly, Agustí et al. (2019) found a fruit-dependent epigenetic regulation of flowering in *Citrus.* The presence of fruit causes epigenetic changes correlated to a flower repressor (*CcMADS19)* that prevents the activation of the floral promoter CiFT2. *CcMADS19* active/repressed state is correlated with changes in histone methylation. Strategies of seed priming through chemical, physical, and biological agents to induce mild stress in the early phases of germination are largely used and rely on an epigenetic basis (Gady et al. 2017; Srivastava et al. 2021). Srivastava et al. (2021) argue that during the seed-to-seedling transition the prevailing stress prime/marks can be imprinted on the genome, resulting in a plant with improved stress tolerance. Nonetheless, much remains to be understood about epigenetic mechanisms involved in seed priming and stress memory.

Complex epigenetic networks regulate fruits' development, both in dry, such as in *Arabidopsis,* and in flesh, with the tomato fruit being the better-studied example (Seymour et al. 2008). Epigenetic mechanisms such as histone modifications, DNA (de)methylation, small RNA posttranscriptional *locus* regulation, and lncRNA-associated regulatory pathway are involved in tomato flower and fruit processes (Gady et al. 2017). Currently, it is known that histone post-translational modifications and the remodeling of the chromatin structure can regulate fruit development and ripening, determining its shape, texture, and color (Gallusci et al. 2016). Also, there are very well-studied cases of how changes in DNA methylation can alter fruit color and the density of trichomes. An example is the Cnr (Colorless non-ripening) tomato mutant, a case in which epialleles determine the existence of a different fruit phenotype due to changes in methylation at a given locus (Gallusci et al. 2016; Nogueira 2019). Besides the mechanisms mentioned, the participation of small RNAs and long noncoding RNAs (lncRNAs) in the different stages of fruit development has been reviewed, but specific functions of most identified RNAs remain to be elucidated (Gady et al. 2017).

In response to injuries, epigenetic changes have also been found throughout fruit development and maturation. Zhang et al. (2016) observed that increases in DNA methylation are related to the loss of flavor induced by freezing in tomatoes. Sicilia et al. (2020) also correlated DNA methylation to increased synthesis of anthocyanins responsible for the blood-red color in certain orange fruit varieties. Since the synthesis and content of metabolites is potentially modified by fruit memory, it is reasonable to suppose the existence of epigenetic control. However, studies that assess whether epigenetic markers generated by a first stimulus could remain and change fruit responses to a second event still need to be explored.

3.2 Metabolic Changes and Enzymatic Modulation

Studies related to the application of a stimulus (i.e., priming the plant or the fruit) usually aim to increase fruit shelf life and improve quality. Once some improvement is found, understanding the underlying mechanisms becomes a fundamental scientific target. When abiotic stimuli are presented, biochemical changes associated with improved fruit performance are: (1) increased amounts of soluble solids, certain amino acids, proline, ascorbic acid (AsA) and glutathione; (2) reduction in electrolyte leakage, malondialdehyde, reactive oxygen species (ROS), often as a result of (3) increases in the activities of antioxidant enzymes, such as catalase and ascorbate peroxidase (Fig. 1b) (Tanou et al. 2017; Wang and Zhu 2017; Zhang et al. 2017). Enhanced gene expression of enzymatic ROS scavengers, such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT), as well as heat shock protein (HSP) genes are also observed (Kang et al. 2001; Wang and Zhu 2017; Marc et al. 2020). In a more in-depth study on cold acclimation of cucumbers, Wang et al. (2018) found that proteins related to defense responses were up-regulated. Lafuente et al. (2017) reported that transcriptomic analysis showed that this class of proteins was more regulated in citrus fruits tolerant to cold injury.

Regarding biotic stimuli, the enhancement of the antioxidant activity is also observed, and it increases the content of phenolic compounds, such as anthocyanin and lignin (Deng et al. 2015; Li et al. 2018; Valenzuela-Riffo et al. 2020). In the same way, higher activities of another group of enzymes are commonly verified, these include β-1,3-glucanase (GLU), phenylalanine ammonia-lyase (PAL), chitinase (CHI), and polyphenoloxidase (PPO) (Yao and Tian 2005; Zheng et al. 2011; Yu et al. 2014; Deng et al. 2015; Shen and Yang 2017; Wei et al. 2018). Activation of the transcription of these key enzymes against pathogens is also commonly seen when gene expression is studied, this includes genes encoding for chitinases, β-1,3-glucanases and polygalacturonase-inhibiting proteins (Yao and Tian 2005; Saavedra et al. 2017; Valenzuela-Riffo et al. 2020). Proteomic studies also have revealed the up-regulation of some proteins involved in stress responses, secondary metabolism, transcriptional regulation, and post-translational modification of proteins (Wei et al. 2018; Li et al. 2019).

Further studies considering several "omics" approaches would help to verify whether physiological and biochemical changes are consistent with the transcriptional expression patterns of several genes and protein abundance, as noted by Tanou et al. (2017). This will allow us to elaborate better approximations for an overall fruit memory model.

3.3 Electrical Memory: Memristors

Volkov and colleagues (Volkov 2016; Volkov et al. 2016; Volkov and Markin 2017) have studied the existence of a hypothetical component of electrical circuits in plants, the "memristors." A memristor is a resistor with memory, which is a passive electrical element of two non-linear terminals that relates the connection of the magnetic flux and the electrical charge. Leon Chua proposed the concept of memristance (memory resistance) at the beginning of the 1970s (Chua 1971). Memristors have already been found in plants, seeds, flowers, fruits, and are supposed to be involved with electrical signaling in plants (Volkov et al. 2016; Volkov and Markin 2017). They depend on the history and state of the system and are possibly associated with the electrical memory of plants. Voltage-gated channels are proposed as biological memristors for both neural networks and plants. The K^+ voltage-gated ion channels are proposed as memresistances in plants as voltage-gated ion channels can regulate the generation and transduction of electrical signals along plasmodesmata and phloem (Fig. 1c). The tetraethylammonium chloride (TEACl) – a K^+ channel inhibitor – was used in experiments with Golden Delicious, Arkansas Black, and Gala apple fruits (*Malus domestica* Borkh.). In addition to the TEACl, fruits were electro-stimulated by cyclic voltammetry and Volkov et al. (2016) found that TEACl inhibited the memristive properties of fruits. These findings are important to create models of electrical circuits in fruits. Also, it is important for a better understanding of any stimuli-processing in fruits, such as memory.

Recently, memristors have been related to short-term memory in the Venus flytrap (*Dionaea muscipula* Ellis). Another experiment also using cyclic voltammetry – bipolar and unipolar periodic electrical waves – showed that the Venus flytrap was able to accumulate small subthreshold charges during 30 s, and when the threshold value was reached, the trap was closed (Volkov and Chua 2021). The authors argued that the cumulative character of electrical stimuli is evidence of the existence of short-term electrical memory. Evidence for memristors in fruits was already found (Volkov and Markin 2017); nonetheless, studies related to short-term fruit electrical memory have not yet been published, being important to the understanding of the modular nature of memory in plants.

Some experimental evidence for fruit memory is discussed in the next section, being important to highlight that such memory can have chemical, epigenetic, and electrical bases (Thellier and Lüttge 2013; Crisp et al. 2016; Souza et al. 2018; Galviz et al. 2020; Volkov and Chua 2021).

4 Evidence for Possible Fruit Memory

4.1 The Challenge of Finding Studies on Memory in Fruits

The existence of memory in fruits has not received much attention in the literature. In fact, few authors come close to point a link between the terms “memory” and “fruit,” and when they do, the memory effect (i.e., previous events changing responses to subsequent stress events separated in time) was not addressed in the experimental design tested. This is the case of Mattoo et al. (2007) and Mattoo and Handa (2008), who wrote openly about memory in fruits, more specifically metabolic memory induced by accumulation of higher polyamides and without application of any stimulus or stress. The aforementioned authors used genetically modified tomatoes for ripening-specific accumulation of spermidine and spermine, which upon reaching a threshold are able to revive the cellular programs underlying N:C signaling, energy and glucose metabolites. This is what the authors refer to as metabolic memory in fruits.

Regarding studies based on different environmental stimulus (biotic and abiotic) or application of chemical elicitors with the ability to change responses to successive stress events, the term memory remains surprisingly absent. On the other hand, terms such as "acclimation," "conditioning," "tolerance," and "resistance" are frequently used (Ballester and Lafuente 2017; Pétriacq et al. 2018; Shi et al. 2019; Zhao et al. 2019) when, in fact, memory is underlying all these phenomena in fruits. It is known that the use of priming agents can affect levels of hormones, signaling mediators, transcription factors, and defense genes/proteins. In a recent review on priming and memory in seeds, the authors argued that both concepts are different sides of the same coin and point out that seed priming with different agents (sodium chloride – NaCl, salicylic acid – SA, β-aminobutyric acid – BABA, selenium, hydrogen peroxide – H_2O_2, cold, magnetic field, among others) can generate long-term memory in plants (Srivastava et al. 2021).

The scientific literature provides examples of what could be considered long-term memory responses in fruit (Zheng et al. 2011; Wilkinson et al. 2018; Reissig et al. 2020). Some other responses could fit within the concept of short-term memory, in a time range spanning from hours to days (Trewavas 2017). An experimental approach in which seedlings or plants are treated/challenged to induce fruit memory would allow us to demonstrate which sort of stimuli experienced by plants can leave marks that modify fruit responses. On the other hand, treatments applied directly to the fruit could support the fact that stimuli can be perceived, processed, and eventually create a memory, which would be perceived and recalled in the future (Saavedra et al. 2017; Valenzuela-Riffo et al. 2020). In this sense, experiments with pre-harvest treatment can more accurately illustrate examples of long-term memory acquired by mother-plants or fruits, while post-harvest treatments are more suitable to refer to a memory of shorter duration.

4.2 *Pre-harvest Treatments Inducing Long-Lasting Responses*

The exogenous application of molecules in plants and fruits has the potential to change fruit resistance/tolerance against several stresses, such as fungi (Wilkinson et al. 2018), bacteria (Song and Ryu 2013; Luna et al. 2020), and cold (Cheng et al. 2018; Shi et al. 2019). Although chemical molecules are not considered stressors, they are able to act as elicitors that trigger the plant defense, resulting in protective responses to biotic or abiotic stresses (Ruiz-García and Gómez-Plaza 2013). Long-term effects of these molecules (time after application) can be observed in treated plants, which maintain a memory of elicitation and respond more quickly to future stress.

For example, the long-lasting effects of BABA application in tomato plants were reported by Wilkinson et al. (2018). BABA application to seedlings significantly reduced post-harvest infection of tomatoes by gray mold (*Botrytis cinerea* Pers.), which was explained through a defense-associated metabolomic profile. More recently, BABA also induced resistance against *Phytophthora infestans* (Mont.) de Bary and *Pseudomonas syringae* Van Hall, 1904 (Luna et al. 2020). L-arginine is another molecule that induces post-harvest resistance to *B. cinerea* in tomato fruit through its effects on nitric oxide biosynthesis and higher activities of phenylalanine ammonia-lyase (PAL), chitinase (CHI), glutamyl endopeptidase (GLU), and polyphenol oxidase (PPO) (Zheng et al. 2011). *B. cinerea* infection induced after 18 days of harvesting was also reduced in Chilean strawberry (*Fragaria chiloensis* (L.) Mill.) fruits by the pre-harvest application of methyl jasmonate (MeJA) (Saavedra et al. 2017). Harvested fruits of *F. chiloensis* showed an up-regulation of genes encoding for the pathogenesis-related proteins (β-1,3-glucanases and chitinases) and for polygalacturonase-inhibiting proteins. Similar results were observed in strawberry fruits, with the MeJA application from the flowering stage onwards priming defense responses after inoculation of *B. cinerea* in harvested fruit (Valenzuela-Riffo et al. 2020).

Enzymatic responses were also observed in muskmelon fruit in response to multiple sprays of acibenzolar-S-methyl (ASM) (a plant hormone-like compound and an analogue of salicylic acid) in whole muskmelon plants from flowering to harvest. Such ASM supplying improved the activity of defense-related enzymes such as peroxidase (POD), PAL, GLU, and CHI, increased phenolic compounds, lignin, and flavonoids levels, reduced latent infection, and induced resistance during post-harvest and storage (Zhang et al. 2011). Pre-harvest applications of abscisic acid (ABA) and MeJA on fruit quality and cracking tolerance of sweet cherry (*Prunus avium* (L.) L.) were also evaluated by Balbontín et al. (2018). Both compounds significantly reduced the number of mature cracked fruit after 6 h of immersion in water and decreased cracking index (−87%) without compromising the fruit weight and diameter.

Alternate abiotic conditions during plant cultivation and fruit development can also affect fruit performance under recurrent stress. For instance, Kang et al. (2001) found that increases in daytime temperature (from 27 ± 1°C to 32 ± 1°C) during cultivation of cucumber plants improved the post-harvest chilling tolerance after 16 days at 10°C. Such improvement was quantified as an increase in the storage life, a reduction in ion leakage from mesocarp disks, an increase in vitamin C content, and higher activities of superoxide dismutase and catalase. Controlled water deficit has also been used for fruit priming. Murshed et al. (2008) showed that mild drought stress applied to tomato plants minimized fruit dehydration, the accumulation of ROS and membrane lipid peroxidation when plants were subjected later to severe drought stress. In "Micro-Tom" tomatoes, Reissig et al. (2020) found that the pre-harvest water deficit delayed the ripening during the post-harvest. Also, fruits from plants that experienced water deficit had a more effective antioxidant defense and reduced oxidative damage. It was observed through lower ROS concentrations, higher catalase (CAT) activity and higher concentration of ascorbic acid and phenolic compounds in the second period of stress (hypoxia). High levels of total phenols and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging persisted until the end of the storage period, demonstrating a long-term response. These findings revealed that the pre-harvest exposure of fruit to water deficit influences the antioxidant system when fruits were subjected to post-harvest hypoxia, which is evidence for memory.

4.3 Post-harvest Treatments Inducing Possible Memory-Associated Responses

Several biotic (fungi, bacteria) and abiotic (heat, cold, low oxygen, UV-C) stimuli are capable of inducing resistance/tolerance during post-harvest. They are used as pre-treatments to reduce the incidence of diseases and injuries caused by storage conditions (Pedreschi and Lurie 2015). An example is the use of low temperatures to acclimate fruits to freezing temperatures. Zhang et al. (2017) pre-treated mango fruits with low-temperature conditioning (LTC) at 12°C for 24 h, then fruits were refrigerated at 5°C for 25 days and chilling injury (CI) was evaluated. Results of this study showed that LTC effectively suppressed the development of CI in mango fruit, such as accelerated softening and increase of soluble solids and proline contents. Biochemical and molecular analysis demonstrated that LTC induces chilling tolerance in mango fruit by physiological and molecular alterations. Wang and Zhu (2017) also confirmed that LTC at 10°C before storage at 5°C improved cucumber fruit quality by impeding ROS accumulation and maintaining cell membrane integrity.

In peach fruits, pre-conditioning at 20°C prior to low-temperature exposure (0°C for 40 days) was effective in suppressing CI symptoms (Tanou et al. 2017). Such findings were explained through changes in the patterns of genes, proteins, and metabolite profiles, resulting in acclimatization to the cold. More precisely, this is achieved by increasing the expression of genes that encode proteins that participate in the biosynthesis of valine, isoleucine, and ethylene, as well as by accumulating higher contents of 1-aminocyclopropane-1-carboxylic acid (ACC) and 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC). Interestingly, Tanou et al. (2017) found increases in isoleucine and valine contents in peach fruits pre-conditioned before cold storage, indicating that specific sectors of primary metabolism may have been altered. The changes in pre-conditioned peach at 20°C resembled metabolic priming associated with cold acclimation, as observed in the changes associated with the amino acid pool size. In view of these results, along with other changes in metabolism, the authors suggest that responses to cold injury "can be sensed differently depending on the pre-chilling history of the peach fruit" (Tanou et al. 2017). In other words, peach memory of a previous event is affecting its future behavior.

Another example of cold acclimation was reported in Granny Smith apples (*M. domestica*), in which the effects of both pre-harvest and post-harvest acclimation were evaluated (Marc et al. 2020). In vivo pre-harvest acclimation to cold was induced by a warm period followed by sudden cold temperatures 3 weeks before harvest. One-week post-harvest pre-conditioning at 8°C before apple storage at 2°C significantly reduced the superficial scald development.

Hot water treatment at 40°C (for 5 or 10 min) decreased infection by *M. fructicola* on peach fruits, as demonstrated by Liu et al. (2012). The hot water treatment was able to induce the gene expression and the activities of CHI, GLU, and PAL. In addition to temperature, the treatment of fruit with LED blue light (LBL) is also a strategy to reduce infection by pathogens. Ballester and Lafuente (2017) demonstrated that previous exposure of sweet orange fruits for different periods at LBL quantum fluxes (ranged between 60 and 630 μmol m^{-2} s^{-1}) induced resistance to *Penicillium digitatum* (Pers.) Sacc inoculated 3 days after light treatment. The results showed that LBL can increase phenylpropanoids concentration and ethylene production in oranges. However, it is necessary to understand how these responses participate in the improved response to *P. digitatum*.

The exposure of harvested cherry tomato fruit (*Solanum lycopersicum* var. *cerasiforme* [Dunal] Spooner, Anderson and Jansen) to *Cryptococcus laurentii* (Kuff.) C.E. Skinner, 1950 induced disease resistance against *B. cinerea* and *Alternaria alternata* (Fr.) Keissl. 1912. Defense-related enzymes CAT, POD, SOD were activated, as well as SA and JA signaling pathways. Also, the up-regulation of various pathogenesis-related proteins was observed, such as GLU, PR-P2, CHI, PR5-like, STH2, and TSI1 (Lai et al. 2018).

So far, we have brought some evidence to support the concept of memory in fruit. As noted, many studies seem to have already suggested its effects, yet much remains to be discovered about the underlying mechanisms linked to fruit memory. At the end, what would be the practical implications of attributing memory to fruit?

5 Perspectives: How to Explore Fruit Memory

Using new approaches to understand the phenomena that occur during fruit growth and development is extremely important to understand the complexity of this plant organ, which plays such an important role in angiosperms. To recognize memory in fruits implies acknowledging that they can store pre- and post-harvest information and use it to better respond to future adversities. In short, perhaps memory is the most important phenomenon behind fruit acclimation to different pre- and post-harvest stress conditions.

Plant homeostasis and resilience are likely related to stress memory. Recently, an index was created that took it into account the overall stability index (OSt) (Ribeiro et al. 2021). An increase in OSt was found either in plants subjected to recurrent stimuli or in propagules obtained from stressed plants, with memory explaining such enhancement of organism stability. Such indexes may also be suitable for fruits as they may help to compare fruits in terms of “memory capacity” by using variables such as respiratory rate and ethylene emission in climacteric fruits.

Fruit responses to stress are closely related to fruit quality, and increases in antioxidant compounds (e.g., phenols, carotenoids, ascorbic acid) may be an example. Increased concentration of antioxidants protects the fruit from oxidative damage and provides food with bioactive properties for human consumption, with the final concentrations being modulated by past events and then linked to fruit memory. In addition to antioxidant compounds, other parameters associated with fruit quality can also be assessed from a memory perspective, and with that, indexes like OSt can be used to reveal fruit memory and explore this concept for improving fruit shelf life and quality.

Assuming that plants and fruits can retain memory for long periods opens up possibilities for using increasingly earlier treatments. Thus, priming-based technologies are alternatives to increase productivity and fruit quality, as well as to extend post-harvest storage. In seeds, the use of priming agents has been related to memory, improving seedling resilience against stress conditions. In fact, seed priming is an important field of research for enhancing crop productivity, collectively termed "primeomics" (Srivastava et al. 2021).

As mentioned in the Sect. 4 "Experimental evidence of fruit memory," despite existing evidence of memory in fruit, we believe that a more specific experimental arrangement directed to address the phenomenon is still lacking. Here, we propose a model to study fruit memory considering the period between stresses (Fig. 2). There must be a complete recovery period between stress-inducing events to characterize a memory effect, by having just the mechanisms remaining that will influence the future responses. It is important to exclude the direct influence of the induction factor, especially when using priming agents of chemical nature that may persist for long periods.

In Fig. 2a we schematized an experimental model to study long-term memory in fruits, with a first pre-harvest biotic/abiotic stimulus followed by a second post-harvest biotic/abiotic stimulus in the fruit. Here, the recovery period between stimulus/stresses occurs with the fruit in the mother-plant, until the second stimulus in the harvested fruit. As an example, a tomato plant in the reproductive stage, predominantly with mature green fruit, can be subjected to a moderate water deficit, which may be accompanied by the decrease of the soil moisture and leaf stomatal conductance, for example. When the fruit is at the breaker stage, it can be harvested and subjected to conditions of low oxygen concentrations (hypoxia) for 3 days and then restored to normal oxygen conditions, for example (Reissig et al. 2020). With the fruits collected at the sampling points, it is possible to carry out the analysis to assess the memory effect. In other words, it is possible to observe if the first event was able to change the responses to the subsequent one. ROS, components of the enzymatic and non-enzymatic antioxidant system, gene expression (related to ethylene biosynthesis, heat shock proteins – HSPs, primary metabolites, DNA methylases, cell wall-associated proteins, and others), epigenetic analysis (DNA methylation, chromatin restructuring and histone post-translational modifications, and noncoding RNA-mediated epigenetic regulation), and many other parameters can be evaluated. In the experimental model we exemplified two recurrent stimuli, however, it would be interesting to have studies with more recurrent stress events or priming agents, to access the extent of the memory generated in the fruit.

Regarding short-term memory (Fig. 2b), both first and second biotic/abiotic stimuli are schematized here in the post-harvest period. The recovery period between stimulus/stresses occurs only in the post-harvest period. The pre-exposure of the fruit to microorganisms followed by a second biotic exposure is an example of an experiment to assess induction of memory and resistance in fruit. To illustrate the example, tomato fruit can be first exposed to the yeast *Cryptococcus laurentii* and

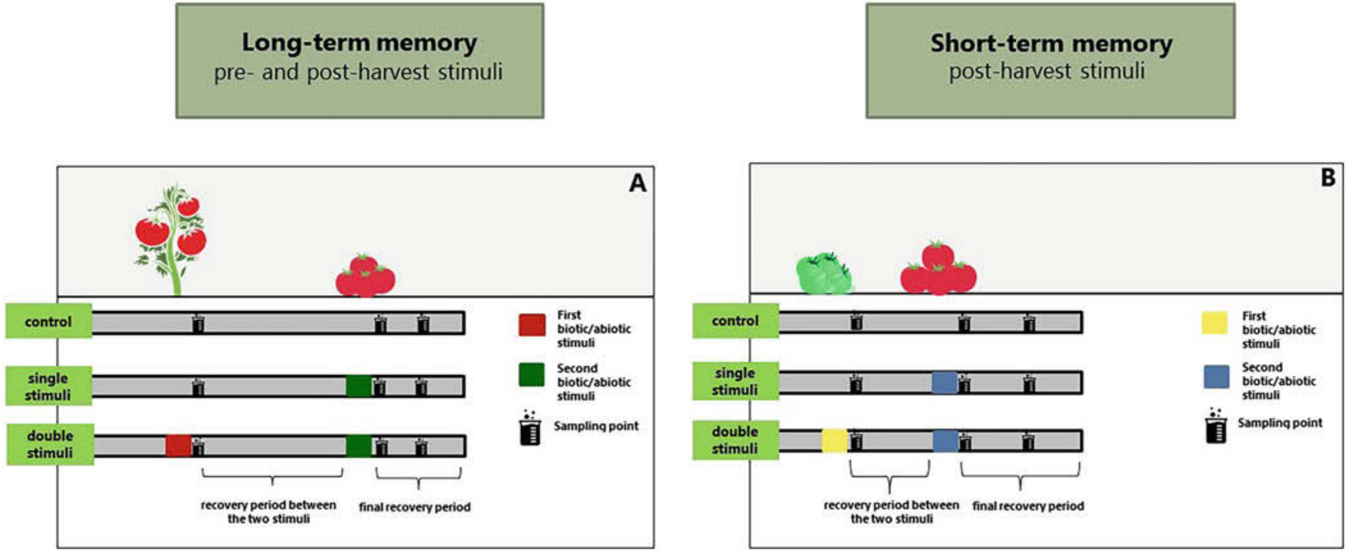

Fig. 2 Experimental model to test memory in fruit. An experiment to test long-term memory in fruit (**a**): A pre-harvest stimulus is applied in the mother-plant. Soon after, a sampling point is required. Before the second stimulus, a recovery period is necessary. The second stimulus is applied to the harvested fruit. Right after the second stimulus, a sampling point is required. A final recovery period and a sampling point are necessary. A post-harvest experiment to test short-term memory (**b**): The harvested fruit is subjected to a first stimulus and soon after the sample is collected for analysis. Before the second stimulus, a short recovery period is necessary. The second stimulus is applied and right after a sampling point is required. A final recovery period and a sampling point are necessary. In both experimental cases, the control (first gray line) and the single stimuli (second gray line) treatment should be conducted concurrently to ensure the study of the first stimulus' memory effect

then, after 2 days, to pathogenic microorganisms *B. cinerea* or *A. alternata* (Lai et al. 2018). In order to assess the responses associated with fruit memory, analysis such as ROS, antioxidant enzymes (SOD, POD, CAT, APX), PAL activity, GLU, activity, CHI activity, defense-related genes expression (MYC2, LOX, JAZ, PR1, NPR1, PR-P2, CHI, PR5-like, STH2, TSI1, PGIP, WRKY33, among others), epigenetic analysis (DNA methylation, chromatin restructuring and histone post-translational modifications, and noncoding RNA-mediated epigenetic regulation), and other parameters can be evaluated.

6 Conclusions

In summary, we presented an overview, discussed some studies evidencing fruit memory and presented experimental models for fruit memory studies. The fact that pre-harvest events induce an improved fruit response during post-harvest "life" is impressive. Also, the fact that a structure detached from the mother-plant can change its metabolism to face environmental constraints is remarkable. Plants are fascinating organisms, and to recognize their most complex and previously unacknowledged aspects is absolutely necessary if we want to blow fresh air into food science and post-harvest technology. We are currently facing unprecedented challenges to feed the world, and innovative approaches for understanding how complex plants and fruits are may bring solutions previously unthinkable.

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Movement of Aquatic Oxygenic Photosynthetic Organisms

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Abstract Many phytoplankton organisms are denser than their aquatic medium, and so sink relative to the surrounding water. Decreasing cell density relative to the medium allows upward movement, using gas vesicles in cyanobacteria and decreasing the density of the vacuole in large-celled eukaryotes. Upward movement can also occur in organisms with flagella. These three mechanisms can maintain the organisms in the photic zone with a small estimated minimum energy cost relative to those of cell growth ($<0.2\%$ in eukaryotic algae and cyanobacteria). When there is limited circulatory water movement in the upper mixed layer the capacity for upward movement relative to surrounding water allows the possibility of periodic vertical migration, making use of opposing gradients of the resources of photon flux density (high near the surface) and nitrogen, phosphorus, and iron (high near the chemocline). For non-flagellate organisms, increased density is needed for the downward portion of the migration. Periodic vertical migration also occurs for flagellates near the surface of muddy or sandy substrates, avoiding excessive light or the possibility of removal in overlying water in tidal habitants. Such movements are also possible in these habitats, and microbial mats on rocky substrata, for cyanobacteria and pennate diatoms with gliding motility. Estimates of the minimum energy cost of periodic vertical migration in these benthic habitats are again a small fraction ($10^{-4}\%$) of the estimated minimum energy cost of growth. High frequency variations in sinking rate of a planktonic marine diatom appear to require a larger fraction of the energy used in cell growth (probably at least 16%) than other mechanisms considered here. The limited data available suggests that the lack of flagella on male gametes of algae (rhodophytes), have little effect on outbreeding and algae diversification. Other dispersal mechanisms (seagrass pollen, wave-driven macrophyte movement) affecting algae outbreeding are discussed.

1 Background

Miyata et al. (2019) propose a phylogeny of motility of organisms, with distinction between active and passive movement. Active movement involves metabolic supply of energy to mechanochemical motors (e.g. flagella: see Appendix) or, in aqueous habitats, regulation of density (Miyata et al. 2019). Passive movement involves prior metabolic inputs to a mechanism that functions after there is no further energy input. Examples are ejection of seeds from the fruits of some legumes upon release of tension produced by desiccation, and wings and parachutes on fruits and seeds (Denny 1993, 2016; Vogel 2020; Miyata et al. 2019; Mann and Lozier 2006). Motion depending on mechanochemical motors potentially, when combined with sensory inputs and signaling pathways, allows directional movement relative to the substratum for movement on surfaces, or for unattached organisms, the moving fluid environments. Motion by buoyancy changes in water only allows movement relative to the force of gravity. Passive movement through fluids does not permit directed movement.

In the case of planktonic photosynthetic organisms, motion relative to the surrounding water using mechanochemical motors (flagella in eukaryotes) or changes in density (ballast; gas vesicles in cyanobacteria, modulation of vacuolar density in eukaryotes) has roles in resource acquisition including movement through the opposing vertical gradient of photosynthetically active radiation and dissolved nutrients such as phosphate and combined nitrogen. For low concentrations of nutrients, motion relative to the surrounding water can increase availability by decreasing the thickness of the diffusion boundary layer. Vertical movement can help to avoid abiotic factors that can limit productivity, e.g. ultraviolet radiation, and biotic factors such as grazers and viruses that remove phytoplankton organisms. Flagellar motility has an important role in sexual reproduction in phytoplankton species with flagellate gametes. Flagellar and gliding motility in benthic microalgae can change position of cells in relation to, for example, the much steeper photosynthetically active radiation gradient in microbial mats and in sediments. Changes in cell density are less significant in benthic microalgae than in phytoplankton. The phenomena discussed in this paragraph are examined in more detail in the body of this paper.

For attached macroscopic organisms, in addition to dispersal by gametes (or structures that carry gametes, e.g. pollen) or products of sexual reproduction, there is also the possibility of lateral vegetative (asexual) spread by elongation growth or detached parts (Collado-Vides 2001). Again, these topics are considered in detail in the rest of the paper.

2 Classification of Aquatic Oxygenic Photosynthetic Organisms in Relation to Vegetative Motility

Luther (1949) and Den Hartog and Segal (1964; see also Raven 1981) proposed a classification of aquatic photosynthetic organisms. Planophytes are unattached to the solid substrate, and include macroscopic benthopleustophytes resting on the substrate, mesopleustophytes between the substrate and the air–water interface, and acropleustophytes floating at the water surface, as well as phytoplankton that are microscopic acropleustophytes (Luther 1949; Den Hartog and Segal 1964; Raven 1981). Organisms attached to substrate are divided into rhizophytes (macroscopic organisms on substrata of small size relative to the organism, and with roots or rhizoids penetrating into the substrata) and haptophytes (microscopic to macroscopic organisms attached to substratum structures that are large relative to the size of the organism) (Luther 1949; Den Hartog and Segal 1964; Raven 1981).

3 Phytoplankton and Benthic Microalgae

3.1 Density and Sinking of Phytoplankton

Phytoplankton are generally denser than their aquatic environment, and so sink under the influence of gravity relative to the surrounding water (Boyd and Gradmann 2002). Turbulence has been shown to increase, rather than, as some have suggested, decrease, sedimentation (Ruiz et al. 2004; see also Ross 2006 and Arrieta et al. 2020). The density differential is larger, other things being equal, in mineralized cells, e.g. diatoms and coccolithophores (Boyd and Gradmann 2002; Raven and Waite 2004). Waite and Harrison (1992) and Waite et al. (1992) showed that metabolic energy is needed for low sinking rates of the centric diatom *Ditylum brightwellii*: illuminated controls sank less rapidly than illuminated cells in the presence of metabolic inhibitors or cells in the dark. Following from this, Waite et al. (1997) show that the predicted (from Stokes' Law, assuming size-independent density) increase in sinking rate of diatoms over a range of cell sizes applies to dead or metabolically inactivated diatoms, but metabolically active cells show lower sinking rates for large-cell organisms than predicted by Stokes' Law. This observation shows that diatoms can use energy to modulate sinking rate through specific physiological mechanisms that decrease cell density, as described in Sect. 3.2. Furthermore, some larger phytoplankton cells can have positive buoyancy, e.g. centric diatoms such as some species of *Coscinodiscus* and *Rhizosolenia*, and also *Ethmodiscus rex*, the non-flagellate vegetative phase of the dinoflagellate *Dinocystis lunaria* and the cyst (phycoma) phase of the prasinophycean chlorophyte alga *Halosphaera* (Boyd and Gradmann 2002; Beardall et al. 2008; Acuña et al. 2010). Changes in cell density of these large cells can result in periodic vertical migration as discussed in the next section.

3.2 Periodic Vertical Migration of Phytoplankton

One important effect of motility of phytoplankton is periodic vertical migration related to the inverse gradients of photosynthetically active radiation (highest at the surface of the water body) and of nutrients (highest at the chemocline near the base of the photic zone) and also any periodic vertical migration of grazing organisms. The mechanism of vertical migration is the vertical component of flagellar motility, and the necessary vertical vector of changes in density of the phytoplankton organism encompassing both higher and lower organism densities than the aquatic medium.

Motility of eukaryotic phytoplankton expressing flagella in the vegetative stage occurs in both marine and fresh water habitats with, among other functions, periodic vertical migration (e.g. Raven and Richardson 1984; Ralston et al. 2007; Hall and Paerl 2011; Bollens et al. 2012; Inoue and Iseri 2012; Schuh and Menden-Deuer 2014). These periodic vertical migrations act to move nutrients to the water surface (Inoue and Iseri 2012).

Occurrence of periodic vertical migration can also occur in cyanobacteria with upward movement when gas vesicles are inflated and the organism density is less than that of the medium, and downward movement when accumulated ballast materials (e.g. polysaccharides) and/or increased turgor deflating gas vesicles causes the density to exceed that of the medium (Walsby 1994). These movements can occur for either marine or freshwater habitats (Walsby 1994). Fogg and Walsby (1971) suggested that periodic vertical migration of cyanobacteria functions in exploiting separation of light and nutrients in the environment of phytoplankton, although Bormans et al. (1999) found little evidence of this in the publications then available. However, Brockes and Ganf (2001; see also Hunter et al. 2008) found density responses of the colonial *Microcystis aeruginosa* to variations in nitrogen, phosphorus, and photon flux density availability consistent with periodic vertical migration. Evidence is consistent with periodic (diel) vertical migration of the diazotrophic colonial cyanobacterium in the central Atlantic (Villareal and Carpenter 1990, 2003), where P (sometime with Fe colimitation) limits N_2 fixation (Sañudo-Wilhelmy et al. 2001; Mills et al. 2004; see also Schlosser et al. 2014; Garcia et al. 2015).

Villareal (1986), Villareal et al. (1999, 2014) showed that nitrate was moved upward in the surface ocean in mats of the diatom *Rhizosolenia.* Wirtz and Smith (2020) show that, in the oligotrophic ocean, long-period (many days) periodic migrations can significantly increase primary productivity, additional to the known effects of diel periodicity migrations, so that vertical pumping of nutrients can be responsible for half of total marine primary productivity, which in turn is almost half of total primary productivity on Earth (Field et al. 1998).

Since fresh water is less dense than sea water, decreasing the density of cells below that of fresh water by means of vacuolar accumulation of solutes yielding very low-density solutions is not possible (Boyd and Gradmann 2002), even with active water transport (Raven and Doblin 2014). Periodic vertical migration in fresh water is, as mentioned above, possible for cyanobacteria with gas vesicles such as the colonial *Microcystis* (Hunter et al. 2008), and for flagellates such as *Volvox* (Sommer and Gliwicz 1986).

3.3 Energetics of Periodic Vertical Migration in Flagellates

Raven and Richardson (1984) calculated the energetics of movement as a fraction of energy used in growth for a marine dinoflagellate; the outcomes are summarized in Table 1. Using on the minimum energy cost of motility based on the work done against friction in flagella operation plus the cost of flagella synthesis, a first-order estimate of the energy cost of motility is 10^{-3} that of cell synthesis (Table 1). Substituting the mechanistic (ATP use) energy cost of motility for the work done against friction, the minimum energy cost of motility (operation plus synthesis of flagella) is 2×10^{-3} that of cell synthesis (Tables 1 and 2).

For freshwater flagellate algae there is also the question of volume regulation. Metabolism requires a higher intracellular osmolarity than the osmolarity of freshwater, so there is a driving force for water entry (Raven 1982, 2000, 2018). The presence of flagella requires that there is an area of plasmalemma not protected from osmotic expansion by the cell wall (if present) (Raven 1982, 2000, 2018). Volume maintenance under these conditions requires active water efflux, typically involving contractile vacuoles (Raven 1982, 2000, 2018). Hence, the energy cost of vertical periodic migration in freshwater flagellates is expected to be higher than that for marine flagellates.

3.4 Energetics of Buoyancy and Periodic Vertical Migration in Cyanobacteria

Walsby (1994) has computed the energy cost per unit time for an *Escherichia coli*-sized cyanobacterial cell with a generation time of 1 day, i.e. a specific growth rate of 0.69 per day, and computed the cost of gas vesicle synthesis during growth as 2.3×10^{-15} W cell^{-1}. Walsby (1994) did not compute the energy cost of cell growth; the calculations that follow are based on the cell properties that Walsby used, i.e. fresh mass of 9.5×10^{-16} kg per cell, and a generation time of 1 day (specific growth of 0.69 per day). Assuming a fresh/dry weight ratio of 3.33, the cell dry weight is 2.9×10^{-16} kg so, with 0.4 g C per g of dry matter, there is 9.7×10^{-15} mol C per cell. From Kliphuis et al. (2012), the absorbed photon cost per cell C during growth is 23 mol photons (400–700 nm); for photochemistry using 680 nm excitation (175 kJ per mol photon) the energy used is 8.89×10^{-8} J per cell. If only 0.3 of this energy is stored in the growing cell, with a generation time of 1 day, this corresponds to $8.89 \times 10^{-8} \times 0.4/24 \times 3{,}600 = 2.3 \times 10^{-15}$ W cell^{-1}

Table 1 Comparison of minimum energy costs of motility allowing periodic vertical migration in phytoplankton as a fraction of the energy cost of cell synthesis for three different means of upward movement. The rates of energy use per cell are related to the specified generation times

	Dinoflagellate	Cyanobacterium	*Ethmodiscus rex* (centric diatom)
Generation time	[a]1 day	[b]1 day	[c]10 days
Cell dry matter	[a]1.64×10^{-11} kg cell^{-1}	[d]2.9×10^{-16} kg cell^{-1}	[e]1.43×10^{-8} kg cell^{-1}
Speed of cell movement	[a]5×10^{-4} m s^{-1}	[b]$2\ 0.10^{-0.6}$ m.s^{-1}	[c]4.6×10^{-5} m s^{-1}
Energy cost of cell synthesis	[a]1.49×10^{-9} W cell^{-1}	[b]4.5×10^{-12} W cell^{-1}	[f]8.9×10^{-7} W cell^{-1}
Energy cost of flagella synthesis	[a]1.28×10^{-12} W cell^{-1}	N/A	N/A
Energy cost of flagella function	[a]0.19–1.38×10^{-12} W cell^{-1}	N/A.	N/A
Energy cost of flagella synthesis + function	[a,g]1.47–2.66. 10^{-12} W cell^{-1}	N/A	N/A
Energy cost of gas vesicle synthesis	N/A	[b]2.3×10^{-15} W cell^{-1}	N/A
Energy cost of active H_2O influx	N/A	N/A	[h]2.42×10^{-8} W cell^{-1}
Energy cost of flagella synthesis and function as a fraction of energy cost of cell synthesis (ratio)	$\leq 2 \times 10^{-3}$	N/A	N/A
Energy cost of gas vesicle synthesis as a fraction of energy cost of cell synthesis (ratio)	N/A	1.3×10^{-3}	N/A.
Energy cost of active H_2O influx as a fraction of energy cost of cell synthesis (ratio)	N/A	N/A	0.016

[a] Raven and Richardson (1984)
[b] Walsby (1994), assuming dry mass is 0.4 of dry mass
[c] Villareal et al. (1999)
[d] From wet mass in Walsby (1994)
[e] Villareal et al. (1999) and Kliphuis et al. (2012) assuming 0.33 of energy in absorbed photons is used in cell growth
[f] Villareal (1999)
[g] Lower value based on work done against friction; higher value from energy consumption in flagella activity
[h] Raven and Doblin (2014), with active water influx only used in 5 days of positive buoyancy

(Table 1). The cost of gas vesicle synthesis is then 1.3×10^{-3} of the cost of cell synthesis. (Tables 1 and 2).

Individual cyanobacterial cells with the characteristics modeled only move upwards at ≤ 2 μm s^{-1} (Walsby 1994; Tables 1 and 2). However, association of cells in colonies can increase the movement speed to ≥ 1 mm s^{-1} based on calculations using Stokes' Law (Walsby 1994). While faster movement relative to

Table 2 Energy of cyanobacterial, dinoflagellate and diatom motility as a fraction of the cost of cell synthesis

Organism, process	Energy cost of movement as fraction of energy cost of growth	Speed of movement	Source
Planktonic dinoflagellate flagella PVM[a]	$\leq 0.00288^{b}$	5×10^{-4} m s^{-1}	Table 1 Sect. 3.3
Plantonic cyanobacterium gas vesicle PVM[a]	0.0013	2×10^{-6} m s^{-1}	Table 1 Sect. 3.4
Planktonic diatom buoyant aqueous vacuole PVM[a]	0.016	4.6×10^{-5} m s^{-1}	Table 1 Sect. 3.8
Planktonic diatom episodic sinking active water influx	0.16[b]	Not applicable	Sect. 3.7
Cyanobacterium in microbial mat or mud/sand	0.005[c]	2.7×10^{-6} m s^{-1}	Sect. 3.9
Raphid diatom gliding in microbial mat or mud/sand	10^{-6c}	10^{-5} m s^{-1}	Sect. 3.9

[a] *PVM* periodic vertical migration

[b] Does not include the cost of synthesis of the catalytic mechanism of active water transport

[c] Does not include the cost of synthesis of the mechanochemical mechanism of gliding motility

surrounding water decreases the boundary layer thickness of organisms of a given size and thus increases solute diffusion to the organism surface and increase nutrient influx, this is partly offset in the case of colony formation by the intrinsically greater diffusion boundary layer around larger organisms (Beardall et al. 2008). Furthermore, colonies have further constraints imposed by the needs for diffusion of nutrients through the matrix to non-surface cells in the colony, as well as increased package (self-shading) restriction on light availability to the average colony cell in a given radiation field (Beardall et al. 2008), as well as diversion of some photosynthate from cell multiplication to producing the extracellular matrix of the colony. How motility at ~0.12 μm s^{-1}, by an unknown mechanism, of cells within colonies of the freshwater cyanobacterium *Microcystis wesenbergii* (Mulling et al. 2014) might address some of these negative aspects of enhanced motility by coloniality is not clear.

3.5 The Swimming Cyanobacterium

Are there alternatives to gas vesicles in allowing upward movement of planktonic cyanobacteria relative to the surrounding water? While no cyanobacteria have bacterial flagella, about a third of open ocean isolates of *Synechococcus* can swim through open water at 5–25 μm s^{-1} (Waterbury et al. 1985; Ehlers and Koiller 2011; Ehlers and Oster 2012). Possible mechanisms of this motility are presented by Ehlers and Koiller (2011) as acoustic streaming based on waves in the (outer?) cell membrane and Ehlers and Oster (2012) as helical waves at the cell surface (outer membrane), driven by mechanochemical motors attached to peptidoglycan in the periplasm. Energization of the outer membrane of cyanobacteria is incompletely understood (Raven and Sánchez-Baracaldo 2021; Rees and Raven 2021).

3.6 Regulation of Sinking Rate of Planktonic Diatoms in Relation to Virus Infection

Raven and Waite (2004) suggested that the interference of virus attack with the ability to decrease cell density of diatoms could cause infected cells to sink faster than uninfected cells, thus limiting spread of the infection. When Raven and Waite (2004) wrote there was very little knowledge of viral infection of diatoms; since then several viruses of marine diatoms (Yuji et al. 2015) have been characterized. While virus infection can convert planktonic particulate organic carbon into dissolved organic carbon (Fuhrman 1999; Wilhelm and Suttle 1999), and thus decrease the extent of the biological pump per unit of primary productivity, Yamada et al. (2018) found that the presence of a virus of *Chaetoceros tenuissima* (3–10 μm equivalent spherical diameter) caused a 5–59-fold increase in the number of cell aggregates 50–400 μm equivalent spherical diameter relative to virus-free control cultures. This aggregation increases the rate of particulate organic matter sedimentation of the cells in the clump, although viral infections may overall increase the rate of conversion of diatom particulate organic into dissolved organic matter (Fuhrman 1999; Suttle 2007).

The influence of virus infection on diatom sinking can be further affected by Si limitation, Kranzler et al. (2019) showed that Si limitation of diatom growth increased viral infection and the resulting cell mortality. Virus infection can also promote the loss of diatoms from the water column via induction of spore production decreasing the possibility of infection of remaining vegetative cells in marine diatoms (Pelusi et al. 2021).

3.7 Energetics of Intermittent Sinking in Planktonic Diatoms

Gemmell et al. (2016) showed that the centric diatom *Coscinodiscus wailesii* showed unsteady sinking. In the light, with periods of rapid sinking alternating with periods of slow sinking with a periodicity of the order of 10 s, when exposed to high nutrient conditions for hours after a period of growth at low-nutrient concentrations. This alternation was shown not to enhance nutrient flux to the cell surface, important under low-nutrient conditions, relative to steady sinking at the same mean rate (Gemmell et al. 2016). Du Clos et al. (2019) showed that the unsteady cell movement was muted when the sinking occurred in the dark. Du Clos et al. (2021) found that there was increasing unsteadiness of sinking of nutrient-limited *C. wailesii* after addition of NO_3^- and $Si(OH)_4$, but not HPO_4^{2-}. They suggested that this unsteadiness may be a response to patchy nutrients, helping to maximize light exposure and minimize energy costs of unsteady sinking. The energy cost of oscillatory sinking was calculated by Lavoie and Raven (2020). These authors showed that the least energy expensive mechanism of unsteady sinking, i.e. episodic cell volume increases with active water influx on the time scale of the periodicity of sinking rate variation, still had a minimum energy cost of 0.16 of the baseline energy cost of growth (Table 2). This energy cost of unsteady sinking exceeds that of generation of buoyancy by gas vesicles or active water influx or upward swimming using flagella (Tables 1 and 2), in periodic vertical migration by over an order of magnitude. To what extent oscillatory sinking is beneficial for diatoms in nutrient patchy environments as well as the physiological mechanisms involved require further investigation.

3.8 Energetics of Buoyancy and Periodic Vertical Migration of Marine Planktonic Diatoms

Some species of *Rhizosolenia* and *Coscinodiscus*, and *Ethmodiscus rex*, are capable of positive buoyancy (Moore and Villareal 1996; Villareal et al. 1999; Boyd and Gradmann 2002; Raven and Doblin 2014; Lavoie et al. 2016). The mechanism for positive buoyancy with the lowest energy cost, i.e. 4.84×10^{-8} W cell^{-1}, is density reduction by active water influx for *E. rex* (Raven and Doblin 2014). With 0.478 μmol C per cell of *E. rex* (Villareal et al. 1999) and 23 mol absorbed photons of photosynthetically active radiation per mol C incorporated into cell material (Kliphuis et al. 2012), cell doubling requires 11 μmol absorbed photons per cell or, for the photons used in photochemistry at 680 nm, 4 J per cell. Assuming 33% of absorbed photons are converted to energy stored in cell material, i.e. 1.32 J, and a specific growth rate of 0.069 per day (Villareal et al. 1999), i.e. cell doubling time of 10 days, the power used in growth is $1.32/10 \times 24 \times 3{,}600$ or 1.52×10^{-6} W. The energy used in active water transport is only used in the ascending leg of the vertical cycling, assumed to take 5 days, active water transport costs a mean 2.42×10^{-8} W cell^{-1}. Buoyancy generation costs then uses 0.016 of the energy used in growth (Tables 1 and 2).

3.9 Periodic Vertical Migration of Benthic Cyanobacteria and Microalgae

Such vertical migration can occur in epilithic or hypolithic microbial mats, including stromatolites and thrombolites (Bebout and Garcia-Pichel 1995; Nadeau et al. 1999;

Consalvey et al. 2004; Lichtenberg et al. 2020), and where the photosynthetic microorganisms occur at the interface of bulk waters and small inorganic plus organic particles of muddy or sandy materials (Round and Palmer 1966; Moss 1977; Paulíčova et al. 2008).

Avoidance of UV radiation appears to be a major function of vertical migration of cyanobacteria in microbial mats in the hypersaline Solar Lake in Egypt (Bebout and Garcia-Pichel 1995) and Antarctica (Nadeau et al. 1999). Consalvey et al. (2004) found the dominant diatom vertical migration in estuarine microbial mats involved downward movement in anticipation of the incoming tide, and upward migration as the tide recedes. Epipelic/epipsamic diatoms and *Euglena* spp. in an estuary move to the surface in the light, constrained by downward movement as the tide comes in (Round and Palmer 1966).

3.10 Energetics of Periodic Vertical Migration by Benthic Cyanobacteria and Microalgae

Raven (1983) calculated the minimum energy cost of gliding motility in such filamentous cyanobacteria as *Oscillatoria* and *Phormidium* at 2.7×10^{-6} m s^{-1} based on work done against friction, suggesting that the energy used was 0.01 of that available from respiration, or perhaps 0.005 of that from photosynthesis, assuming constant movement (Table 2). Marques da Silva et al. (2020) computed the energy cost of vertical migration at 10^{-6} m s^{-1} of raphid pennate diatoms such as *Nitzschia* spp. in a 400 μm photic zone; the small depth of the photic zone is a result of light attenuation by particles in the sediment. Marques da Silva et al. (2020) calculated the minimum energy cost of movement based on work done to overcome gravity and friction, and compared it to the energy stored from photosynthesis. The minimum energy cost of movement over 24 h is only 10^{-6} of the energy stored in photosynthesis over 24 h, granted one vertical migration cycle each 24 h (Marques da Silva et al. 2020) (Table 2).

4 Sexual and Asexual Reproduction in the Absence of Flagella

4.1 Ancestral Sexual Reproduction in Eukaryotes

Genes for meiosis occur in many cultured algae in which sexual reproduction has not been reported, consistent with the occurrence of haploid-diploid alternation typical of eukaryote sexual reproduction (Grimsley et al. 2010; Fučiková et al. 2015). It is possible that the Last Eucaryotic Common Ancestor had sexual reproduction involving flagellate cells, with chemotaxis allowing the gametes to find one another

(Venuleo et al. 2017). As shown in the Appendix, there have been several independent losses of flagellar motility among photosynthetic eukaryotes.

4.2 Spores of Multicellular Red Algae

Pickett-Heaps et al. (2001) measured the gliding motility on surfaces at 0.66 μm s^{-1} of the unicellular *Porphyridium*, and the Chantransia stage spores (10 μm diameter) of the multicellular *Batrachospermum* glide at ~2.2 μm s^{-1}; the latter speed is of the same order of magnitude of speed of cyanobacterial movement (see Table 2). The motility mechanism of algal spores has been subsequently studied by Ackland et al. (2007), who showed that pseudopodia of archaespores of the multicellular alga *Porphyra pulchella* operate using, as expected, actin and myosin.

4.3 Zygnematophyceae

The Zygnematophyceae (=Conjugatophyceae) is the most speciose of the classes of the algal Streptophyta, and has benthic and planktonic representatives (Van den Hoek et al. 1995; Algaebase). Sexual reproduction of these aflagellate (see Appendix) algae involves cells (desmids) or filaments (Zygnematales) of compatible genotypes coming into contact, dissolution of the parts of the cell walls that are in contact, rounding up of cell contents with loss of the aqueous vacuole, and amoeboid movement of the contents of one cell into the other followed by fusion of the protoplasts and formation of a zygospore (van den Hoek et al. 1995). The other classes of algal Streptophyta are the Klebsormidiophyceae (benthic and planktonic), Coleochaetophyceae (benthic), and Charophyceae (benthic) that have flagellate male gametes in oogamous sexual reproduction (van den Hoek et al. 1995).

4.4 Pennate Diatoms (Bacillariophyceae Sensu Stricto)

Although the basal diatoms are the oogamous planktonic centric (Mediophyceae, according to Algaebase) organisms with flagellate male gametes (van den Hoek et al. 1995; Nakov et al. 2018), flagella have been lost at least twice in diatom evolution (Nakov et al. 2018).

One loss was in the ancestor of the pennate diatoms, with basal paraphyletic araphid taxa and derived monophyletic raphid species, i.e. diatoms with a raphe, which is a slit in the cell wall (frustule) (Round et al. 1990; Cox 2012). Most araphid diatoms are benthic, e.g. the araphid *Ardissonea crystallina* that is motile, leaving a mucus trail (Pickett-Heaps et al. 1991). Gametes move using ‘pseudopodia’ and are functionally dimorphic (anisogamous) (Sato et al. 2011; Nakov et al. 2018). Sato et al. (2011) describe mobility of male gametes of the araphid *Pseudostaurosira trainorii* as based on extrusion and retrieval of microtubule-based threads, possibly with kinesin or tubulin forming a mechanochemical motor, with sex pheromones that guide the initially 'random walk' motility? Davidovich et al. (2012) describe related motility of male gametes of the araphid *Tabellaria* spp.

The other loss was in *Ardissonea crystallina*, a member of the benthic marine toxariid clade of centric diatoms (Davidovich et al. 2017). The gametes are functionally dimorphic and have amoeboid movement (Davidovich et al. 2017). There must be close approach of sexually compatible strains not just for the pennate diatoms where the partners become enveloped in a common mucilage sheath before the amoeboid gametes fuse, but also to a lesser extent for centric diatoms with flagellate male and non-motile female gametes (van den Hoek et al. 1995; Nakov et al. 2018).

Notwithstanding the apparent role, from ultrastructural evidence, of microtubules in gliding locomotion of the araphid *Pseudostaurosira trainorii* (Sato et al. 2011), there is substantial evidence (Edgar and Zavortink 1983; Poulsen et al. 1999; Bertrand 2008; Yamoaka et al. 2016) showing that actomyosin is involved in gliding of both raphid and araphid pennate diatoms.

Montresor et al. (2016) review evidence for sex in planktonic marine diatoms. Almost all of the data on the occurrence of sex in marine planktonic comes from work on cultures, with very limited direct data (e.g., observation of empty gametangia) from nature (Montresor et al. 2016). However, there is also evidence of the occurrence of sexual reproduction in nature from molecular genetic data (Montresor et al. 2016). Botte et al. (2013) and Montresor et al. (2016) point out the need for calm water for diatom aggregation and sexual reproduction in planktonic diatoms. Collective sinking of aggregated algae has indeed been shown to promote pairing and reproduction in planktonic pennate diatoms (Font-Muñoz et al. 2019). Diversification rates in diatoms have been related to their locomotion and life history, as are diversification and the extent of outbreeding in the Zygnematophyceae, Rhodophyta, and seagrasses (Nakov et al. 2018; Table 2 of Collins et al. 2013).

5 Vegetative Reproduction and Dispersal of Aquatic Macrophytes

Collado-Vides (2001) reviews the spatially limited lateral dispersal of marine macroalgae by separation of ramets, i.e. genetically identical but physically separate organisms as a mean of vegetative reproduction with little lateral spread. This can occur by heterotrichy (Fritsch 1942), i.e. growth of filaments along the substrate with erect branches, with the possibility of separation of ramets by scission (e.g. by herbivores or abiotic forces) of the horizontal filaments. Heterotrichy occurs in the Rhodophyta: Floridiophyceae (e.g. *Bostrychia*, *Gelidium*), Chlorophyta: Chlorophyceae (e.g. *Stigeoclonium*), and Ulvophyceae (e.g. the acellular *Caulerpa*, *Penicillus*, *Udotea,* and the multicellular *Trentepohlia*), and Ochrophyta: Phaeophyceae (e.g. Ectocarpales) (Fritsch 1942; Collado-Vides 2001). Horizontal growth rate of the stolons of five *Caulerpa* spp. in situ is up to 10 mm per day (Williams et al. 1985), and that of *Caulerpa sertularioides* in the laboratory is 4 mm per day (Mosquera-Murillo and Peñasalanera 2016). As for heterotrichy, scission of the horizontal axis by herbivory or abiotic damage can separate ramets.

Frond growth from a common holdfast for haptophytes (Raven 1981) gives less lateral spread, e.g. in the Rhodophyta: Floridiophyceae (e.g. *Corallina*, *Mazaella*), Chlorophyta: Ulvophyceae (e.g. *Blidingia*), and Ochrophyta: Phaeophyceae (e.g. *Ascophyllum*) (Collado-Vides 2001). Another possibility for ramet separation is branch bending and re-attachment to the rocky substratum followed by separation of the newly attached structure, e.g. *Laurencia* (Ochrophyta: Phaeophyceae) (Collado-Vides 2001). Finally, there is crustose growth with lateral spread and, again, the possibility of separation of ramets, e.g. *Mesophyllum* (Rhodophyta: Floridiophyceae) and *Ralfsia* (Ochrophyta: Phaeophyceae) (Collado-Vides 2001). Matsuda (1989) examined three crustose species of the Floridiophyceae (Rhodophyceae), and found the maximum horizontal growth rate of 0.13 mm per day.

Seagrasses have rhizomes allowing horizontal growth as a mean of vegetative reproduction; the highest value reported by Marba and Duarte (1998) is for *Halophila ovalis* growing at 9.7 mm per day. For comparison with terrestrial vascular plants, Marrs and Watt (2006) report elongation rates of 3.5 mm per day for the fern *Pteridium aquilinum.*

Apart from ramets formed by breaking of horizontal structures discussed in the three preceding paragraphs, macroalgal detachment also contributes to vegetative reproduction and dispersal. Long distance dispersal by detached, viable portions of benthic macrophytes that are exposed directly to the water body is in principle enhanced if the detached portions are buoyant. This has been examined for *Durvillaea* (Fucales: Pheophyceae) which has species with gas spaces in their thallus (e.g. *D. antarctica*) and are buoyant, and species lacking gas spaces (e.g. *D. pomatorum*) (Fraser et al. 2020). Fraser et al. (2020) point out that the occurrence of gas spaces limits the depth at which algae can occur, and also that there have been multiple gains and losses of gas spaces in the genus. Li et al. (2020) examined genetic connectivity among attached populations, and a free-floating population, of the gas vacuolate *Sargassum horneri* (Fucales: Phaeophyceae) in Chinese marginal seas, and found few shared haplotypes between the attached and nearby rafted populations: the rafted population had an unknown origin. Burnett and Koehl (2017) showed that pneumatocysts of *Egregia densa* (Laminariales: Phaeophyceae) provide buoyancy with minimal effect on drag in wave-driven flow. Other things being equal, the occurrence of pneumatocysts provides buoyancy with minimal increase in the possibility of wave-driven detachment. As expected, wounding increased breaking, but rapid growth rate also increased the chance of breaking, of *Egregia densa* (Burnett and Koehl 2019).

De Bettignies et al. (2020) examined survival in situ of fragments of *Laminaria hyperborea* (Laminariales: Phaeophyceae) sporophytes, a kelp that lacks gas spaces.

They found that some fragments maintained photosystem activity after 25 weeks, when only 16% of the original biomass remained. Importantly, some reproductive activity was retained after 20 weeks. It thus appears that despite the absence of gas spaces, reproductive capacity could be important for dispersal granted adequate wave or current activity.

6 Elongate Male Gametes and Pollen Grains in Aquatic Macrophytes

6.1 Rhodophyta

Non-flagellate spermatia of red algae are produced by haploid male gametophytes. Spermatia are surrounded by an extracellular covering and also have two elongate extracellular appendages at 180° (Fetter and Neushul 1981; Brawley and Johnson 1992; van den Hoek et al. 1995; Kaczmarska and Dowe 1997. Engel et al. 1999; Engel 2002; Santelices 2002; Mine et al. 2003; Engel et al. 2004; Maggs et al. 2011). The appendages are believed to increase the chance of encounter of a spermatium in moving water with a trichogyne on a female gametophyte. Destombe et al. (1990) show that spermatia of *Gracilaria verrucosa* remain fertile for 5 h after release, and can fertilize cystocarps 80 m from the spermatia source. Measurements of the fraction of eggs of red algae that are fertilized in the natural environment gave higher values than had been assumed. Santelices (2002) cites 30–80% of eggs fertilized in the brooding-type red algae, which tend to be lower than the 70–100% of broadcast-type fucoid brown algae, but higher than had been assumed. This relatively large fraction of eggs that are fertilized without using flagellar apparatus invites reconsideration of the hypothesis that the carposporophyte phase of the life cycle, producing many diploid carpospores from each zygote is an evolutionary response to a low fraction of eggs that are fertilized.

6.2 Seagrasses

Seagrasses, submerged marine flowering plants, are less phylogenetically diverse than submerged freshwater flowering plants: they are all members of the monocotyledonous order Alismatales. Most seagrasses are rooted in fine-grained substrates, although some (e.g. *Phyllospadix*) grow on rocky shores (Raven 1981; Williams 1995). Movement processes in seagrasses include vegetative spread of clones through rhizomatous lateral growth through the fine-grained substrate or, for *Phyllospadix*, over rocky substrates, drifting of vegetative fragments, and dispersal of sexually produced propagules, and pollen dispersal (McMahon et al. 2014).

The seagrasses are the only flowering plants with submerged pollen release, transfer and pollination (Pettitt 1980, 1981; Pettitt et al. 1980; McConchie and Knox 1989; Cox and Humphries 1992; Reusch 2003; Vermaat et al. 2004; Ackerman 2006; Kendrick et al. 2012; Sinclair et al. 2014). Cox and Humphries (1992) show that 67% of seagrass genera are dioecious, and the remaining 33% are monoecious, although at least 40% of species are monecious, i.e. the monoecious genera are more speciose (Sinclair et al. 2014). Pollen grains of seagrasses are filiform, or functionally filiform by release of spherical grains attached in a filament (Ackerman 2006). Vermaat et al. (2004) used the dioecious *Enhalus acoroides* and showed that there was a large increase in fruit production from carpels (= pollination success) when seagrass cover was over 50%. Moreover, there was no effect of the apparent sex ratio on pollination success indicating that pollen dispersal and pollination success is effective (Vermaat et al. 2004). Sinclair et al. (2014) used the monoecious clonal *Posidonia australis*, and found very high (0.93–0.97 for two sites) genetic diversity in embryos. The pollen dispersal distances inferred from paternity assignment for the two sites are 30.8 and 26.8 m, greater than the mean clonal patch sizes, 12.8 and 13.8 m. Sinclair et al. (2014) tabulate (their Table 4) outbreeding multilocus outcrossing rate reported for monoecious seagrasses; the range is generally 0.61–1.0, with one report of a very wide range of 0.1–0.89. Table 4 of Sinclair et al. (2014) shows a multilocus outcrossing rate of 0.03–0.97 for wind-pollinated terrestrial monoecious Cyperaceae and Poaceae. The data show that the mean multilocus outcrossing rate for the terrestrial wind-pollinated herbaceous plants is less than that for the seagrasses. The quantitative significance of pollination by invertebrate fauna, particularly at night (van Tussenbroek et al. 2016), relative to water movements is not clear. It is certain that the pollen dispersal mechanism in seagrass (without relying on flagella) can lead to significant outbreeding.

7 Conclusions

Movement of organisms or parts of organisms, either active by mechanochemical motors or passively using water movements, are important in the life of aquatic photolithotrophs. Most of these organisms are denser than their aquatic medium, so they, or detached parts, sink relative to the surrounding water in the absence of appropriate mechanochemical motors. Phytoplankton generally sink through their aquatic medium, and retention in the photic zone is not aided by turbulence. Upward movement of non-flagellate organisms is allowed, by decreased density of the aqueous vacuole in large-celled eukaryotes, and gas vesicles in cyanobacteria. Such movement can also occur in organisms with flagella. The small minimum energy cost of these three mechanisms of upward movements (Tables 1 and 2) can maintain the organisms in the photic zone using a small fraction of the energy used in cell growth. When there is limited circulatory water movement in the upper mixed layer there is the capacity for upward movement relative to surrounding water using one of the three upward movement processes. Such movement allows periodic vertical migration relative to the water surface, thus increasing resource gain rate from the opposing gradients of the resources of photon flux density (high near the surface) and nitrogen, phosphorus, and iron (high near the chemocline). Increased density is needed for the downward portion of the migration of non-flagellate organisms, and the periodic vertical migration can only function when there is limited water movement near the surface.

Periodic vertical migration is not limited to phytoplankton; it, and other migrations, can occur in benthic habitats, e.g. for photosynthetic flagellates near the surface of muddy or sandy substrates, moving deeper in avoiding excessive light or in avoiding removal in overlying water in tidal habitats at high tide. Such movements can also occur for cyanobacteria and pennate diatoms with gliding motility in microbial mats on rocky substrata. As for the mechanochemical motility in phytoplankton, the estimates of the minimum energy cost of migration based on work done against friction in these benthic habitats are again a small fraction of the energy cost of growth, although more work is needed to better constrain this energy cost (Table 2). A higher fraction of the energy used in cell growth appears to be needed for high frequency variations in sinking rate of a planktonic marine diatom, but these estimates rely on putative cellular mechanisms that have not been fully resolved yet. More experimental work is thus required to determine the cellular mechanisms and hence refine bioenergetic understanding (Table 2). The limited data available suggests that lacking flagella on male gametes of algae (rhodophytes), have little effect on the extent of fertilization, or of outbreeding, relative to organisms with flagellated male gametes. Other dispersal mechanisms (seagrass pollen, detached portion of benthic macrophytes) are also effective to enhance algae fertilization.

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Appendix. Flagella: What they Are and Where They Occur Among Photosynthetic Organisms

Definition of Flagella

The term ‘flagella’ (singular flagellum) is used for both the bacterial and eukaryotic waggling aqueous motility device. Khan and Scholey (2018) use the terms ‘flagella’ for the bacterial structure, ‘cilia’ for the eukaryote structure, and ‘archaella’ for the analogous structure in the Archaea. However, here the eukaryote organelle is termed, as is it commonly called in plant science, ‘flagella’ (Leadbeater and McCready

2000). It has been suggested that flagella in this sense occurred in the Last Eukaryotic Common Ancestor (Mitchell 2017).

Centrioles/Basal Bodies Are Ancestral

Carvalho-Santos et al. (2011) provide phylogenetic evidence on the ancestral nature of Centrioles/Basal Bodies, and their losses and those of flagella and cilia.

Flagellar and Cytoplasmic Dynein Phylogeny

Wickstead and Gull (2007) showed that cytoplasmic dyneins were lost from Rhodophyta, Viridiplantae, and Entamoeba; flagella dyneins are absent from Rhodophyta and flowering plants.

Fungi: Only Photosynthetic as Lichen Symbioses

Liu et al. (2006) showed that there was only a single loss of the flagellum in fungi: excluding the microsporidia as a sister group to the fungi, the only flagellate fungi are the basal Chytridiomycota. Using a broad definition of symbiosis, some chytrids are parasitic symbionts of algae. Using the narrow definition of symbiosis as mutualistic, Glomeromycotina are mutualistic with embryophytes or (in one case) a cyanobacterium, and Ascomycota and Basidiomycota are mutualistic with vascular plants as mycorrhizas and cyanobacteria and trebouxiophycean and ulvophycean (Trentepohliales) green algae as lichens.

Number of Species With and Without Flagella in the Glaucophyta, Rhodophyta, and Algal Streptophyta

Flagella are common in the Glaucophyta (Jackson et al. 2015) with 25 species (Guiry and Guiry 2021) (15-2-2018). Flagella are lacking in the Rhodophyta with 7,034 species (Guiry and Guiry 2021). The sister group to the Rhodophyta, the non-photosynthetic phagotrophic Rhodelphidia, have flagella (Gawryluk et al. 2019; Colp and Archibald 2019). The algal Streptophyta (=Charophyta) have 5,068 species, of which the flagella-less Zygnematphyceae have 4,150 species, and almost all of the remaining 918 species have flagella (Guiry and Guiry 2021).

Chlorophyta

Palmophyllophyceae, Prasinophyceae, Chlorophyceae, Trebouxiophyceae, and Ulvophyceae

The Palmophyllophyceae and Prasinophyceae are basal Chlorophyta (Leliaert et al. 2012; Leliaert et al. 2016). The known Palmophyllophyceae are benthic palmellmoid organisms; flagella are unknown (Leliaert et al. 2016). The Prasinophyceae are planktonic and have members with (*Micromonas, Mantoniella, Pyramimonas*) and without (*Ostreococcus, Pycnococcus*) expressed flagella (Leliaert et al. 2012).

Chlorella variabilis NC64A (Trebouxiophyceae) is not known to have flagella, but has 103 out of the 360 (29%) flagella-specific proteins in the chlorophycean *Chlamydomonas* (Blanc et al. 2010). Figure 4 of Blanc et al. (2010) compares flagella proteins from *Chlamydomonas reinhardtii* with those of *C. variabilis* NC64A and with *Ostreococcus tauri*, *O. lucimaris, Micromonas* CCMP, and *Micromonas* RCC (Prasinophyceae) and *Thalassiosira pseudonana* (Bacillariophyceae). Of the organisms with flagella, the two *Micromonas* strains had all but one of the 50 *Chlamydomonas* proteins, while *T. pseudonana* only has 16 of the 50 *Chlamydomonas* proteins. The two *Ostreococcus* species each have same 4 of the 50 *Chlamydomonas* proteins. The trebouxiophycean *Asterochloris* has flagellate spores, and has dynein genes (Kellmar 2016).

Merchant et al. (2007) used ‘cilia cut’ to seek *Chlamydomonas* flagellar proteins in organisms known to have flagella (*Homo sapiens*, *Phytophthora* sp.) and those that lack flagella: *Arabidopsis* (angiosperms), *Cyanidioschyzon* (Rhodophyta), *Neurospora*, *Dictyostelium,* and Archaea and Bacteria). *Phaeodactylum* (Bacillariophyceae) lacks flagella genes (Bowler et al. 2008).

The Ulvophyceae are benthic multicellular or coenocytic macroalgae; the reproductive unicells are generally flagellate (Leliaert et al. 2012). This is the case not only for the marine ulvophyceans, and freshwater ulvophycean *Dichotomosiphon* (Moestrup and Hoffman 1975), but also for the subaerial Trentepohliales (Brooks et al. 2015; Zhu et al. 2017). The Chlorophyta have 6,799 species (Guiry and Guiry 2021)

Euglenophyta (Euglenozoa)

These secondary endosymbionts of a green algal endosymbiont in an excavate endosymbiont are universally flagellate. 1,521 species (Guiry and Guiry 2021).

Chlorarachniophyceae (Cercozoa/Rhizaria)

These secondary endosymbionts of a green algal endosymbiont in a rhizarian endosymbiont were first known as amoebae, but some are flagellate. 15 species (Guiry and Guiry 2021).

Alveolata

Dinophyta

Dinoflagellates are apparently ancestrally photosynthetic involving secondary endosymbiosis and, subsequently, tertiary endosymbiosis, but some have lost the capacity to photosynthesize (Raven et al. 2020). Many dinoflagellates are flagellate phytoplankton; others are non-flagellate most of the time, but have a flagellate reproductive phase. 3,490 species (Guiry and Guiry 2021).

Chromerida

Chromerids are photosynthetic symbionts of corals (Raven et al. 2020); the flagellate stage of *Chromera velia* is found more frequently in low salinity cultures (Guo et al. 2010). The chromerid flagella apparatus was modified as part of the host invasion apparatus in their evolutionary descendants, the non-photosynthetic apicomplexan parasites (Woo et al. 2015). 2 species (*Chromera* and *Vitrella*) (Guiry and Guiry 2021).

Ochrophyta

Bacillariophyceae (as Bacillariophyta in Guiry and Guiry 2021)

Vegetative diatom cells are non-flagellate. (Ancestral) centric diatoms have flagellate male gametes; (derived) pennate diatoms have non-flagellate gametes. 16,803 species (Guiry and Guiry 2021). Nakov et al. (2018) relate accelerated diversification in diatoms to their life history and locomotion.

Bolidophyceae

Flagellate planktonic motile cells and non-flagellate cyst (Palmales) phases. 18 species (Guiry and Guiry 2021).

Chrysomerophyceae

Filamentous, with flagellate spores (Kai et al. 2008). 7 species (Algaebase).

Chrysophyceae

Flagellate planktonic unicells or colonies; some filaments or macroscopic (*Hydrurus*) non-motile benthic organisms with flagellate reproductive phase. 764 species (Guiry and Guiry 2021).

Dictyochophyceae

Marine flagellate planktonic cells. 161 species (Guiry and Guiry 2021).

Eustigmatophyceae

Coccoid freshwater (a few marine) cells; reproductive cells flagellate. 102 species (Guiry and Guiry 2021).

Pelagophyceae

Marine plankton, coccoid or flagellate; some benthic palmelloid or filamentous. 25 species (Guiry and Guiry 2021).

Phaeophyceae

Alternation of phases, except in the Fucales. Motile spores, except in Dictyotales. Both gametes flagellate in isogamous/anisogamous species, male gamete flagellate in oogamous species. 2,061 species (Guiry and Guiry 2021).

Phaeothamniophyceae

Filamentous with flagellate spores (Bailey et al. 1998). 35 species (Guiry and Guiry 2021).

Pinguiophyceae

Planktonic, with flagellate stages (Kawachi et al. 2002). 5 species (Algaebase).

Raphidophyceae

Plankonic or psammophilic flagellates in fresh, brackish or marine habitats. 40 species (Guiry and Guiry 2021).

Schizocladiophyceae

Marine, branching filaments: flagellate zoospores. Kawai Mn Maeba et al. (2003). 1 species (Guiry and Guiry 2021).

Synchromophyceae

There are no reports of flagella in the Synchromophyceae (Schmidt et al. 2015). 7 species (Guiry and Guiry 2021).

Synurophyceae

Planktonic flagellate unicells or colonies. 417 species (Guiry and Guiry 2021).

Tribophyceae (Xanthophyceae)

Unicellular, multicellular or (*Vaucheria*) coenocytic. Some vegetative cells are planktonic flagellates. Reproductive cells flagellate; compound zoospores in *Vaucheria*. 695 species (Guiry and Guiry 2021).

Haptophyta

Marine (e.g., coccolithophores, and the colony-forming Phaeocystis), and a few freshwater, plankton, many with flagella. 936 species (Guiry and Guiry 2021).

Cryptophyta

Freshwater and marine planktonic flagellates. 218 species (Guiry and Guiry 2021).

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An Overview of Water and Nutrient Uptake by Epiphytic Bromeliads: New Insights into the Absorptive Capability of Leaf Trichomes and Roots

Cassia Ayumi Takahashi, Antônio Azeredo Coutinho Neto, and Helenice Mercier

Contents

Abstract The presence of specific morphological structures, such as the formation of a tank and the presence of leaf absorbing trichomes, together with peculiar adaptive strategies to capture resources in the epiphytic environment, can be considered one of the most important characteristics that explain the occupational success of several species of bromeliads in the forest canopy. In view of the great importance that specialized nutrition represents for epiphytic bromeliads, this chapter gathers the most recent findings regarding the strong influence that ontogenetic stages and some abiotic factors, such as nitrogen nutrition, can exert on the anatomical formation, absorptive capability and distribution of trichomes present on the leaf surface. Studies showing the existence of water inward transport systems involving high- and low-affinity membrane transport proteins and aquaporins are also discussed. Furthermore, we present new discoveries about how the process of water uptake occurs through the entire anatomical structure of the trichome, highlighting the relevance of the wing cells of the shield. Important advances in recent studies regarding the physiological function of the roots of epiphytic bromeliads are also highlighted. Contrary to what was believed in the past regarding the

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roots of these plants, considering them no more than holdfast structures, the roots of some species of epiphytic tank-forming bromeliads have shown to play an important role in the processes of absorption and metabolization of resources. The new findings related to (1) the influence of developmental stages on the absorptive capability of the roots of epiphytic tank-forming bromeliads, (2) the independence of the absorption mechanisms exerted by the trichomes and roots, and (3) the synergy between the processes of resource uptake carried out by the root system and leaf absorbing trichomes are also presented and discussed in this chapter.

1 Introduction

Throughout evolution, some members of the Bromeliaceae family developed several specific morphological structures and very peculiar adaptive strategies that allowed them to live in the epiphytic environment (Benzing 2000). Such specialized mineral nutrition has been considered, according to Benzing (2000), one of the most important adaptive characteristics for the emergence of epiphytism and the successful occupation of various regions of the canopy by many bromeliad species.

In the forest canopies, bromeliads are able to capture resources from peculiar sources. Water and mineral elements can come from external sources (dry atmospheric deposition, precipitation, fog, mist, dew, gases, and direct contact with clouds) and from internal sources (stemflow, throughfall, decomposition of organic debris, and symbiotic or asymbiotic associations with other organisms, such as diazotrophic bacteria, fungi, insects, amphibians, etc.) (Zotz 2016). Most of the mineral salts and other compounds present in the canopy usually become available to epiphytic bromeliads after being previously diluted in water (Benzing 1990). For this reason, the availability of resources (nutrients and water) in the epiphytic environment is closely linked to the intermittence of rainfall periods and the durability of each individual rainfall occurrence (Zotz 2016). In general, in the first moments of the rain, the initial concentrations of the mineral elements present in the stemflow and throughfall are usually high due to wash-off of dry atmospheric deposition previously accumulated on the leaves and trunks of the trees (Levia et al. 2011; Zotz 2016). This high concentration of nutrients in the water running down the trees usually decreases exponentially as the rainfall period extends over time (Levia et al. 2011; Zotz 2016).

The amount and type of salts dissolved in rainwater vary according to many factors, such as the season of the year, the vigor of the host tree, the relative leachability of mineral elements from different species of trees tops and the proximity of urban regions and marine habitat (Benzing and Renfrow 1974; Clarkson et al. 1986; Benzing 2000). In some regions (e.g., the forest of central Amazonia), the concentration of nutritional resources in the canopy does not exceed a few parts per million (lower than that found in soils) (Benzing 2000), while in other areas (e.g., the lowland forests in Costa Rica or in Panama), the nutrient availability on the branches covered by a soil-like substrate (organic debris + bryophytes) is equal to or greater than that found in forest soil (Zotz 2016).

The leaves are considered the main organs of absorption and metabolization of resources in epiphytic bromeliads. They are often exuberant, well developed, and plentiful, making up the major part of the bromeliad's vegetative body. The peculiar morphological, anatomical, and physiological characteristics of the leaves that comprise the structure of the shoot part of epiphytic bromeliads allow these plants to (1) generate their own "soil" by impounding the organic debris and water into the tank and close to their absorption organs and (2) enable them to capture nutritional resources quickly when the rainwater and leachates briefly pass over their vegetative bodies (Benzing and Renfrow 1974). One of these important characteristics is the existence of special structures, called absorbing trichomes, on leaf surfaces that increase the leaf permeability to take up water and nutrients through the shoot part (Benzing and Burt 1970). Leaf absorbing trichomes (LATs) have been extensively studied for decades regarding their morphology, anatomy, resource absorption function, morphological diversity, and various taxonomic aspects (Benzing 2000). The importance of these absorbing appendages for epiphytic bromeliads can already be considered unquestionable based on the wealth of knowledge accumulated to date. Currently, researchers are deepening their studies of LATs involving (1) the physiological and molecular aspects of membrane transport proteins, (2) the influence exerted by the shield cells with well-developed flexible wings to harvest water rapidly and transport it on the shoot surface, and (3) the role of thick shield walls in the mechanism of resisting water evaporation and reducing water loss in dry environments.

Unlike the leaves, the roots of epiphytic bromeliads are often lignified, reduced in extent and little in abundance. For decades, the root system of these plants was described as a mere holdfast structure that no longer performed the absorptive function (Benzing 2000). However, new findings have shown that the root system of some bromeliad species of this type of bromeliad is physiologically active and participates in the processes of absorption and metabolization of nutrients and water.

This chapter aims to review the most recent discoveries regarding the capture of resources such as water and nutrients (mainly nitrogen (N)) by the foliar trichomes of epiphytic bromeliads. We also include the recent data about the role of the roots of epiphytic bromeliads in the process of resource absorption and metabolization.

2 Leaf Absorbing Trichomes (LATs): General Morphology and Anatomy

The LATs make an important contribution to water and nutrient absorption in epiphytic bromeliads, and in some cases the function of photoprotection has also been observed (Zotz 2016). It is believed that the absorptive function originated from the early-diverging terrestrial bromeliads that occupy oligotrophic habitats (Males 2016).

Precipitation over forest canopies can reach epiphytic bromeliads as throughfall and stemflow carrying many types of nutrients which can be intercepted by the leaves and roots (see also Sect. 3). As rainwater can vary significantly spatiotemporally in the epiphytic habitat, "short water supplies" can be taken up quickly and efficiently through the trichomes, which partially or totally cover the leaf surfaces, facilitating the survival of epiphytic bromeliads in harsh environments (Benzing 1990).

The LATs of bromeliads are multicellular structures present in the leaf epidermis comprising a living stalk and a shield with dead cells (Benzing 2000). Great morphological diversity of LATs is found in the Bromeliaceae family. Members of the subfamily Tillandsioideae show the most complex trichomes structure, comprising (1) a shield formed by a central disc, ring and wing cells and (2) the stalk (the living part), which is made up of the dome and foot cells (Benzing 2000).

Another important morphological feature of some epiphytic bromeliads is the tank, which is formed by the partial overlapping of the leaf bases, creating a series of watertight catchments that enable the accumulation of rainwater and organic debris among the leaves (Benzing 1990). Some tank bromeliads during their ontogenesis have an initial phase, called the atmospheric phase, during which plants lack the tank. The developmental change from juvenile atmospheric to adult-tank form is an example of heteroblasty, which is very common in the subfamily Tillandsioideae (Zotz et al. 2011). The development of the tank structure allowed the epiphytic bromeliads to have a more "continuous supply" of nutrients and water (Benzing 1990).

The leaves of many juvenile atmospheric plants have a uniform trichome distribution along the leaf blade, while the mature tank leaf often shows a longitudinal gradient with the basal portion more densely covered with trichomes, which progressively are reduced in quantity in the apical direction (Takahashi and Mercier 2011; Rodrigues et al. 2016). In the case of *Guzmania monostachia*, an epiphytic tank-forming bromeliad, the specialization of trichome structure is established during the tank development (Kleingesinds et al. 2018). Juvenile atmospheric plants of *G. monostachia* (Fig. 1) show the trichome shield with only one ring and a smaller number of wing cells compared to tank-adult plants (Kleingesinds et al. 2018). The cellular complexity of the trichome shield is modified as the foliar basal portion increases the cell number and the structure of the tank is formed. In the trichome of the adult leaf, two outer rings appear which present a high amount of pectin, indicating an increase in hygroscopic capability and consequently higher competence for rapid water transport and absorption (Boanares et al. 2018). In addition, histochemical evidence obtained for leaves of *G. monostachia* surprisingly indicated the presence of living cells in both ring and wing cells of trichome shield, which is currently reported as being comprised of only dead cells. This finding may suggest a possible absorptive function exercised by shield cells (Kleingesinds et al. 2018).

More and more studies are focusing on foliar water uptake in plants other than bromeliads, attracting a great deal of attention due to its important role in plant

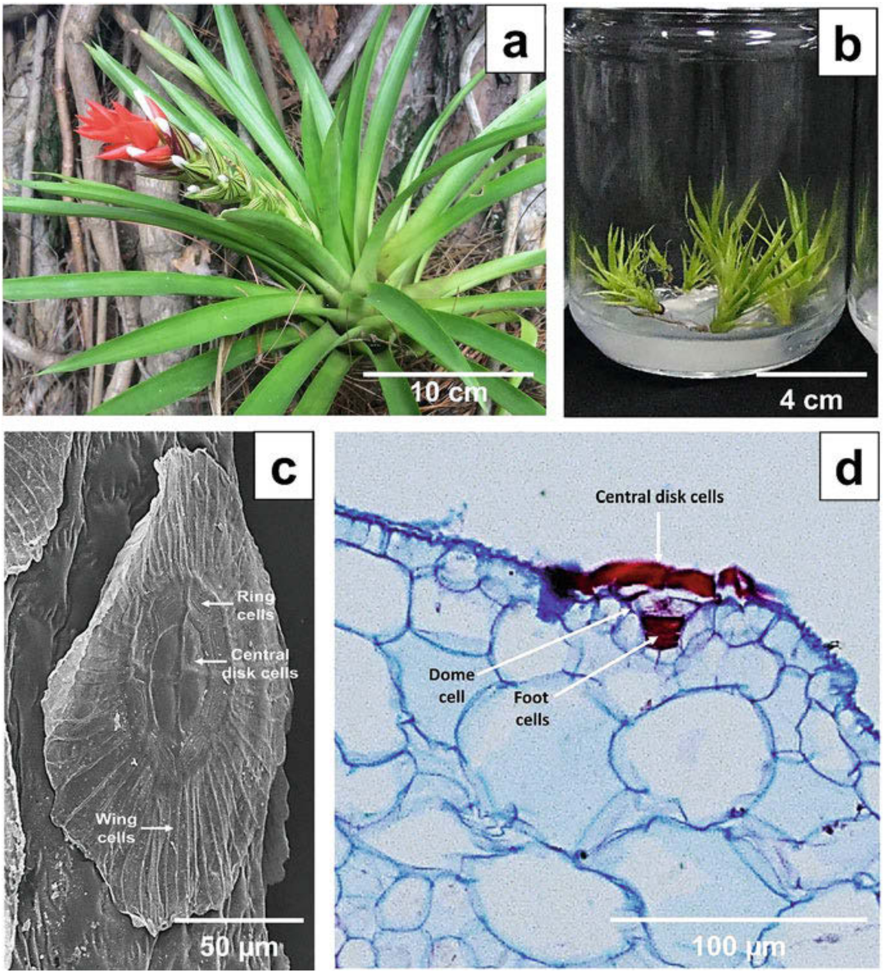

Fig. 1 Plant morphology and leaf anatomy of *G. monostachia*. (**a**) Tank-forming bromeliad in the adult stage; (**b**) juvenile atmospheric plants cultivated in vitro; (**c**) scanning electron micrograph of a LAT from leaf of juvenile atmospheric plant showing the central disk with four cells, the ring with one cell row (total of eight cells) and the wing cells; (**d**) transmitted light micrograph – cross-section – of a LAT from juvenile atmospheric plant showing central disk, dome cell and foot cells (total of two cells)

response to climate change, which may cause increases in temperature and intensify periods of drought (Boanares et al. 2021; Schreel and Steppe 2020). For epiphytic bromeliads, the LATs are considered an important adaptive feature that allows these plants to maintain their water balance and also acquire nutrients from wet deposition. In the next two sections, more details and new findings about the physiological functions of LATs are presented.

2.1 Nutrient Uptake

Meisner et al. (2013) showed the first evidence that during the tank development of heteroblastic bromeliads, the LAT capability can gradually change, modifying its physiological properties or changing the number of transport proteins available on the cell membranes of these leaf appendages as seen in the case of the phosphorus transporters. Zotz (2016) reported the presence of low-affinity and high-affinity transporters in the leaves of tank bromeliads, indicating that the ions can be effectively absorbed from both diluted and more concentrated solutions. More recent work has proposed that the leaf base of adult *G. monostachia* plant is important not only for N uptake, but also for nitrate reduction and urea hydrolysis (Gonçalves et al. 2020). The highest gene expression of high-affinity transporters for nitrate (*NRT*2.5), ammonium (*AMT1.2*), and urea (*DUR3*) was detected in the tank, besides an aquaporin transporter urea (*TIP2.1*), suggesting that the localization of high-affinity transporters in the basal part of the leaves provides an efficient N absorption even at low concentrations, thus improving the chances of this bromeliad to survive during periods of nutrient scarcity (Gonçalves et al. 2020). On the other hand, the leaf apex more intensively expressed low-affinity N-transporters and glutamine synthetase (*GS*) genes (Gonçalves et al. 2020). Interestingly, earlier transcriptional analysis performed with *G. monostachia* leaf already indicated that the greener portions (apex and middle) have differentially expressed genes related to amino acid metabolism, while the base is more related to nutrient uptake and trichome development (Mercier et al. 2019). Due to the fact that the high-affinity transporters are usually found in roots of terrestrial plants, it has been suggested that the basal leaf portion of adult-tank bromeliads may have incorporated some functions of roots of terrestrial plants (Gonçalves et al. 2020).

Different environmental conditions can lead to intraspecific morphological variation (Zotz 2016). For instance, data from our laboratory showed that for juvenile atmospheric plants of the heteroblastic *G. monostachia*, there was a great modification of some trichome traits depending on the N nutrition. NO_3^-, NH_4^+ or urea can cause substantial shifts in the diameter of the trichomes, the wing cell length and the interval distance among them when the small bromeliads were cultivated in vitro (Fig. 2). In comparison to NH_4^+ and urea, NO_3^- increased the diameter and wing cell length of the trichomes in both adaxial and abaxial surfaces and in both foliar parts (basal and apical) while decreasing the interval distance among these leaf appendages (which is more easily seen in the region of leaf base) (Fig. 2a, b). Interestingly, the NO_3^- seemed to influence the size of the water storage tissue (adaxial hydrenchyma of mesophyll) since the plants fertilized with NO_3^- were shown to have the largest thickness of the storage tissue compared to bromeliads which received other N-sources (data not shown). This result suggests that the morphology of the trichome may be important for its capability to absorb/transport a greater or lesser amount of water inside the foliar tissues. Comparing the density of trichomes, NO_3^- and NH_4^+ produced similar results in both portions and leaf surfaces. On the

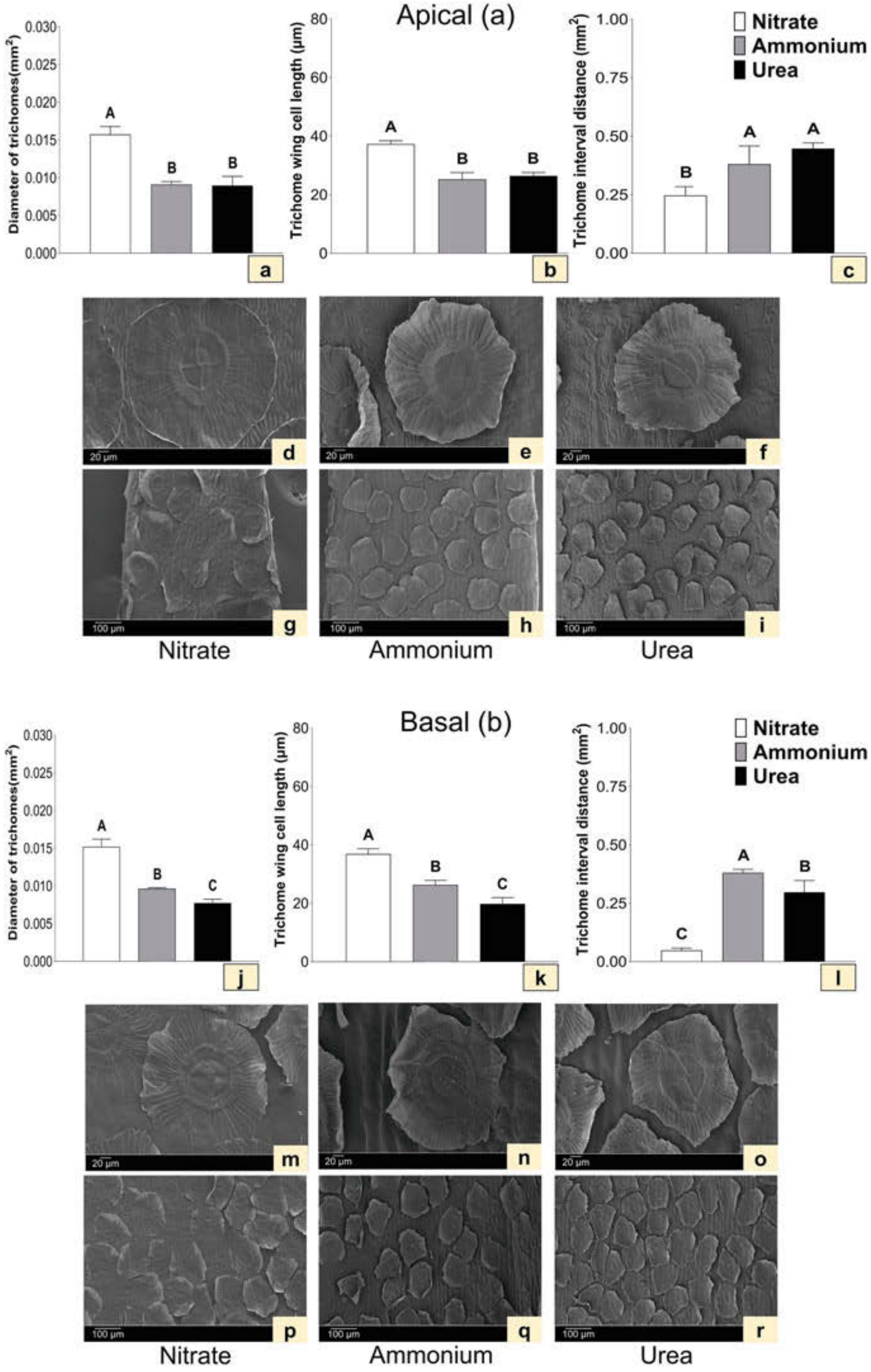

Fig. 2 Comparison between apical (**a**) and basal (**b**) regions of the adaxial leaf surface, showing trichome diameter (a/j – mm^2), wing cell length (b/k – µm), trichome interval distance (c/l – mm^2), and scanning electron microscopy images (d/m, e/n, f/o – 20 µm, g/p, h/q, i/r – 100 µm) of

other hand, urea caused a significant reduction in this parameter (Coutinho Neto 2017).

In the natural environment, nitrate and ammonium can be the N-forms that are usually found diluted in the wet deposition (Van Stan II and Pypker 2015). Areas of the canopy which are heavily covered by adult plants of *Tillandsia usneoides* showed that this atmospheric bromeliad captures more NO_3^- than NH_4^+ from the throughfall. As a consequence, the concentration of this inorganic N-source (nitrate) that reaches to the soil is significantly reduced (Rosier et al. 2015). Atmospheric bromeliads are able to absorb N dissolved in rainwater as it flows over the surface of their vegetative body, while tank-forming bromeliads are able to retain the nutrients leached inside the tank, in addition to organic debris (leaves and branches of the host tree) and organic compounds, such as urea from the excreta left by some animals, e.g., amphibians (Gonçalves et al. 2016; Leroy et al. 2016). For the epiphytic tank-forming bromeliad *Vriesea gigantea*, there is evidence that different types of aquaporins (e.g., plasma membrane intrinsic proteins, PIP, and tonoplast intrinsic protein, TIP) are responsible for N uptake depending on the ontogenetic phase. In juvenile atmospheric plants, the leaf can uptake NH_4^+ by VgPIP1;2 but is not able to absorb the intact urea molecule. On the other hand, the leaf of adult tank-forming bromeliad showed the capability to take up NH_4^+ and the intact molecule of urea by VgTIP2;1 (Matiz et al. 2019). This difference may be the reflection of a physiological change that occurs during the leaf development that allows it to efficiently absorb the N compounds that become more abundant when the tank is formed in the adult phase (Matiz et al. 2019). A controlled experiment using isotope tracer (^{15}N) on ^{15}N-capture showed that NH_4^+ and urea contributed more than NO_3^- to *G. monostachia* nutrition in the adult phase when the tank is completely formed. The leaves presented a greater abundance of $\delta^{15}N$ in the basal part of leaves where high-affinity transporters of ammonium and urea can be activated in the adult stage (Gonçalves et al. 2020).

From fundamental research on epiphytic bromeliads, which face nutritional scarcity in their natural environment, it is possible to discover some peculiar processes involved in nutrient absorption by LATs, revealing possibilities of bioengineering crops through the inclusion of transporters and/or aquaporins with higher affinities for specific nutrients.

Fig. 2 (continued) G. monostachia plants in the juvenile atmospheric stage cultivated in vitro and in the presence of nitrate, ammonium, or urea as sole N-source. A thin water film covers the whole leaf surface when the plants are cultivated in an aseptic culture medium. Aseptic in vitro technique ensures that the N form supplied does not change due to processes carried out by microorganisms, such as nitrifying bacteria that transform NH_4^+ to NO_3^- or other microorganisms that cause urea hydrolysis through the action of urease enzyme, which releases ammonium as a product of this reaction. Similar values were obtained for abaxial leaf surface. Mean values + SDs (standard deviation). Letters indicate statistical differences among treatments with N-sources (ANOVA/ Tukey HSD, $\alpha = 0.05$). ImageJ (Version 1.53n) was used for measurements

2.2 Water Uptake

Leaf permeability to water has an important ecological significance through the interception of atmospheric moisture in the natural environment (Zambrano et al. 2019). Comparing four species of adult atmospheric *Tillandsia* species, it was observed that the structure, arrangement, and density of LAT had a great influence in the liquid spreading and the wettability of the leaf surfaces, consequently improving the foliar water uptake of the plants (Zambrano et al. 2019).

Most atmospheric adult bromeliads, mainly those of the genus *Tillandsia*, whose leaves are not able to form tanks and lack functional roots, have a very dense and homogeneous trichome cover on the entire leaf blade that is capable of spreading water over the epidermal surface, facilitating water distribution and flow. The physiological importance of rapid transport of external water to increase the absorption is clear for bromeliads that occupy xeric microhabitats (Herppich et al. 2019).

Mist and fog may be an important water source for epiphytic plants in forests subjected to seasonal droughts. The film of water that accumulates on the leaves in fog conditions is critical to keep the plant's water balance (Wu et al. 2018). During drier conditions, the tillandsia's trichome wings are kept elevated, but this position shifts when the relative humidity increases in the atmosphere. The water film pulls the wings and flattens them, which indicates the flexibility of these structures (Ha et al. 2021).

For adult plants of *Tillandsia usneoides*, it was verified that water transport occurs mainly by capillarity among overlapping trichomes and not by internal transport through xylem. This atmospheric bromeliad inhabits xeric environments, and low shoot xylem hydraulic conductivity leads to a substantial decrease in leaf water losses (Herppich et al. 2019). A more recent study led by Ha and collaborators (2021) using the same atmospheric species showed the great importance of the flexible wing arrays for the effective and rapid water transport via trichome wings on the leaf surface. The overlapping of adjacent wings plays a bridge-like role that allows the transfer of water to the next wing, resulting in domino-like water transport. Subsequently, the water penetrates the cavities, releasing air bubbles, and the inward absorption takes place through the plasma membrane of the foot cells directed by osmotic potential gradient (Ha et al. 2021). Transmembrane water flow may be regulated by the aquaporins (water channel proteins) that are located in the plasma membrane (PIPs), the tonoplast (TIPs), or in other cellular membranes (Chaumont et al. 2005). For the atmospheric epiphyte *Tillandsia ionantha*, soaking plants or leaves in water stimulated the expression of *TiPIP2*a mRNA (Ohrui et al. 2007). Together with other experiments also performed by these researchers, the increase of aquaporin transcripts indicated the participation of this transmembrane protein in a rapid water uptake through LATs in response to water stimulus.

Interestingly, measurements of water exchanges in *Tillandsia aeranthos* and *Tillandsia landbeckii* showed a great water flux asymmetry, i.e., 5,800-fold asymmetry in overall leaf conductance between absorption and evaporation, which was attributed to trichome structure: juxtaposition of a thick hygroscopic wall (shield) and a semipermeable membrane of the foot cells (stalk) (Raux et al. 2020). The *Tillandsia* species is able to take up fine water droplets during intermittent fog events while also avoiding evaporation in drought conditions. When the trichomes of this species are exposed to high air humidity, the shield walls absorb the water droplets, forming a continuous path of liquid water from the trichome to the cytoplasm of the foot cell. In contrast, in drought conditions, the liquid–gas interface moves deep into the trichome, facilitating the outward water movement by diffusion of water vapor through the thick shield walls rather than capillary flow of liquid water (Raux et al. 2020). Therefore, it has been suggested that the evolutionary innovation of the *Tillandsia* LATs reflects the achievement of a low outward conductance instead of a higher conductance to take up liquid water.

The fundamental research employing biological surfaces on water transport and absorption through flexible trichomes is being conducted to inspire new insights into hydrophilic industrial applications in the fields of engineering, biology, and microfluidics (Zambrano et al. 2019), such as the creation of water harvesting devices like shape-morphing materials in soft robotics (Ha et al. 2021).

3 The Absorptive Capability of Epiphytic Tank-Forming Bromeliad Roots

There is evidence that the ability to take up resources by the roots of tank-forming epiphytic bromeliads varies according to the ontogenetic stage of plant development (Vanhoutte et al. 2017). One of the most recent studies carried out in our laboratory with *V. gigantea*, a tank-forming epiphytic heteroblastic bromeliad, showed that the roots of this plant play a very important role in the processes of absorption and assimilation of some N sources (such as nitrate and urea), especially during the early stages of its development.

The ability to absorb nitrate and urea by plants can be checked through the quantification of the enzymatic activities of nitrate reductase (NR) and urease, respectively, since these enzymes are usually stimulated in the presence of their substrate (nitrate reduction by NR and urea hydrolysis by urease). After supplying a nutrient solution to the root system, the roots of young atmospheric bromeliads of *V. gigantea* are able to rapidly absorb the nitrate source (within 24 h) and efficiently reduce it in the root tissues through the action of NR (high NR activity in roots after root fertilization) (Fig. 3a). One part of the total nitrate absorbed by the roots is transferred to the shoot, where it is reduced by the foliar NR (high NR activity in leaf tissues after root fertilization) (Fig. 3a), showing that juvenile atmospheric *V. gigantea* plants are able to optimize their ability to reduce nitrate through the metabolization of this compound in a large part of their vegetative body (expressive increase in NR activities in leaves and roots after root fertilization) (Fig. 3a). The roots also show a high potential to absorb and hydrolyze urea since urease is a constitutive enzyme in root tissues with expressive activity (Fig. 3b). Similar to

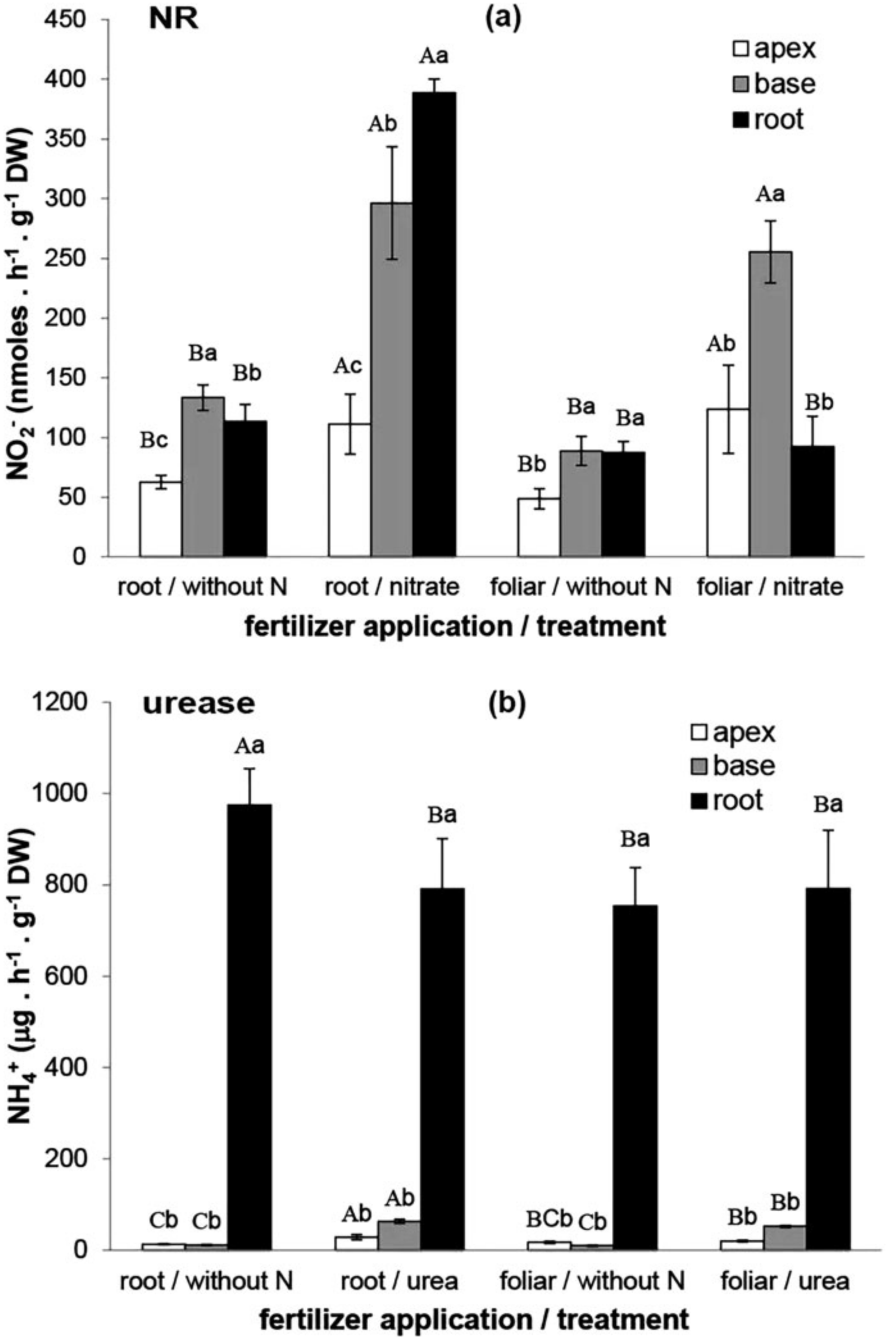

Fig. 3 Activities of nitrate reductase (**a**) or urease (**b**) in two regions of leaf (apex, base) and root of juvenile atmospheric bromeliad *V. gigantea* at 24 h after being supplied with a nutrient solution containing NO_3^- or urea as the unique N source (5 mM of total N) or with a nutrient solution depleted of N sources. Plants previously cultivated in mix substrate (pine bark + commercial

nitrate, one part of the total urea absorbed by the roots is also transferred to the shoot part, where it is hydrolyzed (increase of urease activity in leaf tissues after root fertilization) (Fig. 3b). The leaves of juvenile plants are also very important organs in the processes of absorption and metabolization of nitrate and urea (increase of NR and urease activities in the leaf tissues after foliar fertilization, mainly in the basal region of the leaves). However, roots proved to play a much more important role than leaves in the process of metabolizing these two N sources (higher NR and urease activities were detected in the roots).

In adulthood, the roots of *V. gigantea* are no longer the protagonists in the process of nitrate and urea uptake. NR and urease activities remain at baseline values even after root fertilization (Fig. 4). After the complete formation of the tank structure, the leaf bases become the main organs of absorption and metabolization of these N sources (expressive increase in NR and urease activities measured in the leaf base after addition of the nutrient solution inside the tank) (Fig. 4). These changes of functions between leaves and roots in juvenile and adult bromeliads of *V. gigantea* probably occur gradually throughout the plant development as has been reported for the cultivars *Vriesea* 'Splenriet' and *Vriesea* 'Galaxia' (Vanhoutte et al. 2017).

In the juvenile stages when the structure of the tank begins formation (developmental stages: (1) juvenile with onset of formation of a small tank → (2) adult with well-developed tank → (3) adult with well-developed tank and inflorescence), the roots of the cultivars *V.* 'Splenriet' and *V.* 'Galaxia' are very efficient organs for capturing resources from the substrate to the point of being able to contribute significantly to the total amount of resources absorbed by the young bromeliads (Vanhoutte et al. 2017). The absorptive capability of the roots of juvenile bromeliads gradually decreases in the subsequent stages of ontogenetic development. On the other hand, the capabilities of resource uptake of leaf trichomes intensify throughout the developmental stages (Vanhoutte et al. 2017). In the adult phase, when the tanks of *V.* 'Splenriet' and *V.* 'Galaxia' are already fully developed, the trichomes at the base of the leaves become the main structures for absorbing resources, while the roots with low absorptive capability only complement the nutrient uptake carried out mainly by these leaf appendages (Vanhoutte et al. 2017).

The great importance of the root system for juvenile bromeliads of *Vriesea* cultivars (*V.* 'Splenriet', *V.* 'Galaxia', *V. gigantea*) may be related to two factors: (1) high demand for resources (water and nutrients) and (2) absence or small volume of the tank that is still in formation (Vanhoutte et al. 2017). In the early stages of

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Fig. 3 (continued) organic substrate, 1:1) were transferred to sand substrate 4 days before the start of experiments, in order to deplete, at least in part, the endogenous N. Plants were watered with distilled water every 2 days. After this period, the nutrient solutions were applied exclusively through foliar or root fertilization. The foliar fertilization was done by spray application, while the root fertilization was done by the addition of the nutrient solutions on sand substrate. After 24 h, samples of leaf and roots were used in enzymatic assays. Different capital letters indicate an average significantly different among treatments for each plant material (ANOVA, Tukey-HSD test; $P < 0.05$). Different small letters correspond to an average significantly different among distinct regions of the plant body in each treatment (ANOVA, Tukey-HSD test; $P < 0.05$)

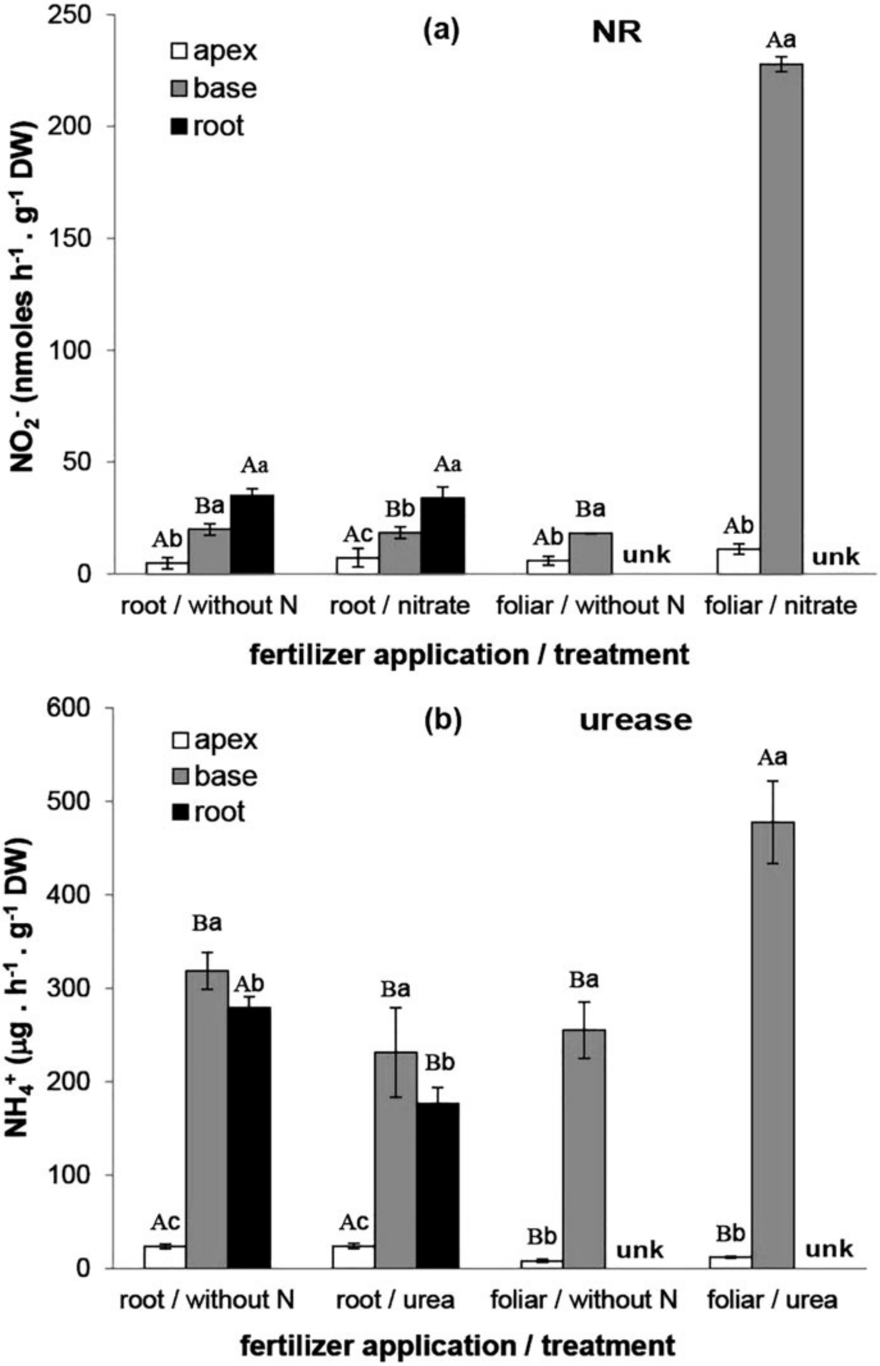

Fig. 4 Activities of nitrate reductase (**a**) or urease (**b**) in two distinct portions of leaves (apex, base) and root of adult epiphytic tank bromeliad *V. gigantea* at 24 h after being supplied with a nutrient solution depleted of N sources or with a nutrient solution containing NO_3^- or urea as the unique N

development, tank-forming epiphytic bromeliads seem to have a high demand for nutrients and water, probably because these plants are in continuous growth at this phase of their life span. The intense demand for nutritional and water resources of juvenile bromeliads can even exceed the demand of resources required by adult bromeliads, as has already been seen in *V.* 'Splenriet' and *V.* 'Galaxia' (Vanhoutte et al. 2017). As young bromeliads of *Vriesea* cultivars (*V.* 'Splenriet', *V.* 'Galaxia') are still very inefficient in capturing and accumulating resources through their leaves, the presence of functional roots is essential for these plants to be able to take up sufficient amounts of nutrients and water to promote their growth and development at satisfactory rates (Vanhoutte et al. 2017). We believe that, when working together with leaves, the functional roots help to increase the total absorption surface of the plant body, allowing the juvenile bromeliad to optimize its abilities to absorb and metabolize nutrients, as seen in the case of *V. gigantea* fertilized with nitrate or urea (Figs. 3 and 4).

Other recent studies have also shown that the roots of some tank-forming epiphytic bromeliads grown in greenhouse continue to be physiologically active even into adulthood (Vanhoutte et al. 2017; Carvalho et al. 2017; Da Silva et al. 2018; Leroy et al. 2019; Gomes et al. 2021), despite the fact that these roots no longer act as protagonists in the process of water and nutrient absorption as seen in young bromeliads (Vanhoutte et al. 2017). In addition, the resource uptake through leaf trichomes or by the root system of adult epiphytic tank bromeliads seem to be two distinct and independent absorption mechanisms with little interference with each other. According to Vanhoutte et al. (2017), in the adult cultivars of *V.* 'Splenriet' and *V.* 'Galaxia', when the uptake rates through leaf trichomes increase greatly due to a higher availability of resources inside the cistern, the absorption capability of the roots does not suffer any type of interference from the intense absorptive action of the trichomes, suggesting that there may not be immediate feedback between the absorption mechanisms exerted by the trichomes or roots. Carvalho et al. (2017) also believe that leaf trichomes and roots have distinct absorption mechanisms that act independently of each other. According to Carvalho et al. (2017) and Proença and Sajo (2008), the roots of adult epiphytic tank bromeliads are anatomically very similar to those of terrestrial bromeliads, suggesting that the root system of these epiphytic plants has the morphology and

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Fig. 4 (continued) source (5 mM of total N). Plants previously cultivated in pots with mix substrate (pine bark + commercial organic substrate, 1:1) were transferred to sand substrate, kept in this cultivation condition for 7 days and watered with distilled water every 2 days. After this period, plants were fertilized with nutrient solution through foliar or root fertilization. The foliar fertilization was done by the addition of the nutrient solution inside the tank, while the root fertilization was done by the addition of the nutrient solutions on sand substrate. After 24 h, samples of leaf and roots were used in enzymatic assays. Different capital letters indicate an average significantly different among treatments for each plant material (ANOVA, Tukey-HSD test; $P < 0.05$). Different small letters correspond to an average significantly different among distinct plant materials in each treatment (ANOVA, Tukey-HSD test; $P < 0.05$). Unknown value (unk) the anatomical structure necessary to perform the absorptive function independently, exactly as the roots of terrestrial plants are able to do.

Furthermore, the roots of some epiphytic tank bromeliads in the adult stage seem to be able to absorb sufficient amounts of water and nutrients to promote plant growth and development. After a nutrient solution containing ammonium nitrate is offered exclusively inside the tank or restricted to the roots, the leaf trichomes and the root system of *Aechmea fasciata*, an adult epiphytic tank bromeliad, are able to efficiently capture this N compound, resulting in significant increases in leaf N content and in the number of newly developed leaves (Gomes et al. 2021). In *Nidularium minutum*, an adult tank-forming bromeliad with terrestrial/saxicolous/epiphytic habits, the roots have the ability to take up water and nutrients as efficiently as the leaf trichomes, resulting in a significant growth of this bromeliad (Carvalho et al. 2017). Interestingly, fertilization occurring strictly inside the tank or exclusively in the roots results in plants with very similar morphologies, i.e., there are no differences between fertilization treatments regarding various growth and development parameters, such as number of leaves, fresh mass (leaves, roots, stem and total), and dry mass (leaves, roots, stem, and total) (Carvalho et al. 2017).

In most adult epiphytic tank bromeliads whose roots are shown to be physiologically active, the absorptive capability of the root system does not surpass the absorptive capability of the leaf trichomes, i.e., the root absorption is usually equal to or less than the uptake rate performed by trichomes. The trichomes of *A. fasciata* showed evidence that they are still the main absorption organs of the plant, although their roots also efficiently perform the uptake of N sources (Gomes et al. 2021). Foliar fertilizations with nutrient solutions containing organic or inorganic N sources result in *A. fasciata* plants with greener leaves and a much more pronounced silver appearance than bromeliads fertilized exclusively on the roots (Gomes et al. 2021). Higher foliar N content was also detected in *A. fasciata* plants that receive the nutrient solution among their leaves (Gomes et al. 2021). Furthermore, the nitrogen use efficiency is higher when the fertilizations with N sources occur inside the cistern than on the substrate (roots) (Gomes et al. 2021).

The root system of *Aechmea aquilegia*, an adult epiphytic tank bromeliad, has a very efficient absorptive capability. The amount of water and nutrients absorbed by these roots is enough to maintain growth and development rates similar to those seen in well-hydrated *A. aquilegia* bromeliads (watering done to leaves and roots) (Leroy et al. 2019). Although the root system of *A. aquilegia* plays this important role in the process of resource uptake, the absorptive capability of the roots does not surpass the absorptive efficiency of the leaf trichomes (Leroy et al. 2019). According to Leroy et al. (2019), the absorptive capability of the roots of *A. aquilegia* varies according to the type of resource that will be absorbed. In the case of water, trichomes and roots have very similar absorptive capabilities, so much so that the water status (i.e., leaf relative water content, leaf water potential, and leaf osmotic potential) of plants watered only on the roots is similar to that of plants that receive water exclusively inside the tank. On the other hand, roots are less efficient in absorbing N sources ($NH_4{}^{15}NO_3$ and $^{15}NH_4NO_3$) than trichomes. Fertilization restricted to the roots with these N compounds results in bromeliads that have a lower foliar N content when compared to plants fertilized solely inside the cistern (Leroy et al. 2019). The differences in water or nutrient uptake capabilities observed between trichomes and roots of *A. aquilegia* suggest that perhaps the biochemical properties of membrane transporters may be different between these two absorption organs (Leroy et al. 2019). Leroy et al. (2019) argue that this hypothesis still needs to be investigated and believe that the process of resource uptake by epiphytic tank bromeliads may be better understood when the functioning and regulation of membrane transport proteins are investigated in greater depth.

Although the roots of epiphytic tank bromeliads in the adult stage are no longer the protagonists in the process of resource uptake, the root system usually acts synergistically with trichomes to absorb water and nutrients available in the environment. As a consequence, there is a significant increase in the total resources absorbed by bromeliads, improving their growth and development rates. In *A. aquilegia*, the uptake of N compounds ($NH_4{}^{15}NO_3$ and $^{15}NH_4NO_3$) is maximized only when fertilization occurs in both absorption organs of the plant, i.e., trichomes and roots (Leroy et al. 2019). Leroy et al. (2019) also observed that leaf N content and ^{15}N isotopic abundance are higher in *A. aquilegia* plants that receive double fertilization (trichomes + roots). This synergistic effect between foliar and root fertilization is also seen in the case of *N. minutum* (Carvalho et al. 2017). When trichomes and roots work together in the process of resource uptake, there is a greater increase in several growth and developmental parameters when compared to plants fertilized only on leaves or roots (Carvalho et al. 2017). In addition, along with the leaf trichomes, the physiologically active roots of the cultivars *Guzmania lingulata* and *Vriesea* 'Harmony', both commercial varieties of epiphytic tank bromeliads, also significantly contribute to bromeliad nutrition, especially in terms of increasing plant biomass (Da Silva et al. 2018).

4 Conclusion

Novel morpho-physiological and molecular researches about leaf absorbing trichomes demonstrate that atmospheric and tank-forming bromeliads achieve a successful way to transport water rapidly through flexible wing arrays facilitating the absorption of water and N by aquaporins and high-affinity transporters which ensure an efficient uptake system at low nutrient concentration. Recent studies show relevant advances in the area of nutrition in tank-forming epiphytic bromeliads to the point of expanding our knowledge and significantly changing our understanding of the role of roots for these plants. We believe that studies of the root system of epiphytic bromeliads are just beginning and a wide range of questions involving this subject will certainly be answered in the near future. We are convinced that more data on these issues will allow researchers to reach a better understanding of the importance of root functionality for the plasticity of bromeliads living in the epiphytic environment.

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Phi Thickenings: Their History, Current Status and Role(s) in Mechanically Strengthening the Plant Root

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Contents

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Progress in Botany (2023) 83: 363–402, https://doi.org/10.1007/124_2020_51,

Abstract Phi thickenings, lignified bands of secondary cell wall encircling root cortical cells, align between adjacent cells to form a complex network that frames the endodermis and central stele. Since their description in numerous angiosperms and gymnosperms in the two decades from the 1860s, there has been little research into the functions of these enigmatic structures. Their cage-like organisation led to speculation that phi thickenings mechanically strengthen the root, but more recent ideas include that they regulate biotic interactions or control ion movements in a manner similar to the Casparian strip. There is, however, sparse direct evidence supporting these suggestions. In this review, we focus on the roles that phi thickenings might play within roots, and although we conclude that the primary function of phi thickenings is to mechanically stiffen the root apex, we emphasise that phi thickenings need not have a single role and that they can be "re-tooled" to perform other functions. We describe several experimental systems for studying phi thickening functions. *Geranium* and *Pelargonium* roots, in which phi thickenings form in cortical cells immediately under the epidermis, are well suited for cell biology investigations, whereas the large aerial roots of epiphytic orchids are ideal for investigating root biomechanics. For genetic analysis, however, the differential induction of phi thickenings in *Brassica* roots in response to water stress or hormones provides a powerful experimental platform to identify regulatory mechanisms and directly test our models of phi thickening functionality. Since phi thickenings typically form in the root apex, we propose that these structures function primarily to strengthen or stiffen the plant root. We reject the concept that phi thickenings function to block ion flows in a manner analogous to the Casparian strip, but instead see the potential for limiting ion flows within the root cortex as drawback to the presence of thickenings. This negative outcome due to the presence of phi thickenings may explain why phi thickenings are only induced in response to specific biotic or abiotic challenges in some species and are not formed constitutively. In some instances, however, phi thickenings may have been "re-tooled" to perform other roles including blocking uptake of ions, notably in the case of Brassicaceae species growing under extreme conditions.

1 Introduction and a History of Phi Thickenings

Phi thickenings are typically thin bands of lignified, secondary cell wall that form around the radial cell walls in the root cortex, a cellular location where only a thin, primary cell wall would normally be found. This narrow band of thickenings encircles the entire cortical cell, and as the thickenings are typically coordinated between adjacent cells, an entire network of lignified thickenings surrounding the central stele is produced. However, unlike the endodermis, phi thickenings do not become suberised, and unlike secondary cell wall development in xylem, the cells that form phi thickenings (phi cells) do not undergo programmed cell death. This review will discuss the possible functions of these phi thickenings within the root.

1.1 Van Tieghem and His Discovery of Phi Thickenings

In 1871, the French botanist Van Tieghem published a long monograph in *Annales des Sciences Naturelles Botanique* on the structural symmetry of vascular plants (Van Tieghem 1871). In a section of gymnosperms (p. 187), he provided the first generally acknowledged description of structures that are now known as phi thickenings, although this term was not one that Van Tieghem himself used:

> L’avant-dernière assise corticale possède un caractère spécial. Ses cellules, à section carrée ou hexagonale, ont au milieu de leurs faces latérales et transverses une bande d'épaississement saillante en dedans et arrondie, et les bandes des cellules voisines se correspondent exactement. Cette bande d'abord blanche, et devenant plus tard jaune clair, forme un cadre rectangulaire qui donne beaucoup de solidité à la cellule.

This statement translates as:

> The penultimate cortical layer [that is, the inner cortical layer] has a special character. Its cells are square or hexagonal in cross section, and have in the middle of their lateral and transverse walls a thickened strip that is rounded and projects into the cell. The strips of the neighbouring cells correspond exactly. This band, initially white, and later becoming light yellow, forms a rectangular frame which gives a lot of solidity to the cell.

Van Tieghem reported these thickenings in a range of different gymnosperms, including *Taxus baccata* (European yew) which he illustrated in cross section, and other species from the Cupressaceae (cypresses) and Taxaceae (yews). In a subsequent series of papers in 1887 and 1888 (Van Tieghem 1887a, b, c, 1888a, b; Van Tieghem and Monal 1888), he also described thickenings in a range of different angiosperm families including the Brassicaceae (the crucifers or cabbage family), the Rosaceae (rose family), the Caprifoliaceae (honeysuckles), the Fabaceae (the legumes), and the Geraniaceae (geraniums). Further, by using clearing and the cell wall stain basic fuchsin, he demonstrated that these cell wall thickenings were lignified. In many cases, Van Tieghem’s work on phi thickenings in a particular species, genus, or family remains the only published description of these structures in those taxa.

1.2 Nicolai and the Discovery of Phi Thickenings

Contrary to statements in all publications concerning phi thickenings since the 1920s, Van Tieghem was not the first to describe these enigmatic structures. In a general study of root development, Nicolai (1865) described cortical cell wall thickenings in gymnosperm roots including *Taxus*, writing on page 62 that:

> die innerste Rindenschicht in eine Schutzscheide verwandelt. Bald nachdem dieses geschehen ist, sieht man in den anderen Zellreihen der Rinde, und zwar zunächst in der Zellreihe, die die Schutzscheide umgiebt, eine eigenthümliche Verdickung, die durch einen verholzten und sich allmälich sehr stark verdickenden, senkrechten Streifen der radialen Wand hervorgerufen wird.....
>
> Ausser bei Coniferen sah ich eine ähnliche Verdickung nur bei *Pyrus malus*, hier aber auf den Zellkreis beschränkt, der unmittelbar die Schutzscheide umgiebt.

In translation:

> the innermost bark [cortical] layer is transformed into a protective sheath [the endodermis]. Shortly after this has happened, one sees a peculiar thickening in the other cell rows of the cortex, first in the cell layer that surrounds the endodermis, which is caused by a lignified and gradually thickening, vertical strip of the radial wall.
>
> Except for conifers, I saw a similar thickening only in *Pyrus malus* [apple], but there it is limited to the ring of cells that immediately surround the endodermis.

Later in the same article, he also described similar, albeit more complex thickenings in the aerial roots of the orchid *Cattleya*. Although cited by Kroemer (1903) and Scott and Whitworth (1928), this initial description of cell wall thickenings seems to have been forgotten. However, while illustrations were not provided, the written descriptions are consistent with the development of phi thickenings in gymnosperm roots, with their initial development in the inner cortex immediately outside the endodermis and subsequent formation of smaller and slightly more random thickenings in layers further out. Moreover, as suggested by Nicolai (1865), phi thickenings also occur in *Pyrus malus* in a single ring in the inner cortex (Riedhart and Guard 1957; Mackenzie 1979; Peterson et al. 1981), while large and complex phi thickenings are present in the aerial roots of the orchid *Cattleya* (Brundrett et al. 1988) and in the related genera *Laelia* (Idris et al., *manuscript in preparation*) and *Rhyncholaelia* (Collings, unpublished data). Thus, it would seem that credit for the initial descriptions of phi thickenings should belong to Nicolai (1865) who not only provided the first description of these structure in gymnosperms, but also gave the first description of similar structures in angiosperms.

1.3 The Origin of the Name “Phi Thickenings”

The term “phi thickenings” was coined neither by Van Tieghem nor Nicolai. In a monograph on the vascular system of plants, Russow (1875) wrote:

> Hierher gehört die in den Wurzeln der Cupressineen und Taxineen vorkommende, zuerst von van Tieghem beschriebene Scheide, welche dadurch ausgezeichnet ist, dass die zur Oberfliche des Centralcylinders rechtwinklig stehenden Wände der sie zusammensetzenden Zellen mit einem planconvexen Verdickungsband versehen sind. Um diese merkwürdig gebaute Scheide, zum Unterschiede von anderen Aussenscheiden, kurz zu bezeichnen, wollen wir sie Φ-Scheide nennen, weil der Querdurchschnitt der verdickten Wand dem griechischen Buchstaben Φ gleicht.

This statement translates as:

> This description includes the protective layer of cells found in the roots of the Cupressaceae and Taxaceae, first described by van Tieghem, which are distinguished by the semi-circular cell wall thickenings in the cell walls that run at right angles to the surface of the central cylinder [stele]. To briefly describe this strange structure, in contrast to other thickened cell walls, we might call them Φ thickenings because the cross-section of the thickened wall resembles the Greek letter Φ.

Russow went on to describe similar phi thickenings in a range of angiosperms from the Rosaceae, Caprifoliaceae, Fabaceae, and Berberidaceae (barberries), but as with Van Tieghem's catalogue of species that contain phi thickenings, the phi thickenings in many of the species that Russow identified have not subsequently been studied.

1.4 The Late Nineteenth Century: A Golden Age for Phi Thickening Discovery

Considering the relative paucity of phi thickening research in the years since 1900, there was a surprising variety of research into these structures in the two decades years immediately after Nicolai, Van Tieghem, and Russow's initial observations. These included similar observations of phi thickenings in a range of gymnosperm species by Klein (1872a, b, c), Reinke (1873) and de Bary (1877), observations of phi thickenings in *Brassica oleracea* roots (Woronin 1878) and observations made by Schwendener (1874) in his book *Das mechanische Princip im anatomischen Bau der Monocotylen* (*The Mechanistic Principle on the Anatomical Structure of Monocots*). In a section entitled "*Mechanically-effective parenchyma cells*" (page 159), he wrote:

> Als mechanisch wirksame Elemente und zwar als Einrichitungen gegen radiale Druckkräfte betrachte ich endlich auch die Zellen der Schutzscheide in Wurzeln und Rhizomen, jedoch nur soweit sie verdickte Wandungen besitzen; ebenso jene eigenthümlichen Membranverdickungen, welche zuweilen im Parenchym ausserhalb der Schutzscheide auftreten und um diese letztere ein zusammenhängendes Netzwerk mit longitudinal gestreckten Maschen herstellen. Wo solche Verdickungsleisten unter der Epidermis oder sonst im Parenchym vorkommen, mögen, sie einem analogen Zwecke dienen. Aber auch hier ist es bloss eine hypothetische Ansicht, die ich ausspreche; die wissenschaftliche Prüfung und Durchführung derselben würde eingehendere Untersuchungen voraussetzen, als ich sie bis dahin angestellt habe.

This translates as:

> Finally, I consider the cells of the endodermis in roots and rhizomes as mechanically effective elements, namely as devices against radial pressure forces, but only as far as they have thickened walls. Likewise, the peculiar cell wall thickenings, which sometimes occur in the parenchyma outside the endodermis, and around which they form a coherent network with a longitudinally elongated meshwork. Wherever such thickenings occur under the epidermis or in the parenchyma, they may serve an analogous purpose. But here, too, this is just a hypothesis view that I am expressing; the scientific examination of this concept would require more detailed investigations than I have so far completed.

Schwendener contributed the first and most direct early statement about a possible mechanical role for the phi thickenings but more than a century later, we are still waiting for “more detailed investigations” to be conducted into the mechanical effects played by phi thickenings in plant roots.

1.5 The Scope of This Review

As we near the 150th anniversary of Van Tieghem’s initial observations of phi thickenings and of the observations by Nicolai, Reinke, de Bary, Russow, Klein, Schwendener, and Woronin, it is worth considering how far we have progressed in understanding these enigmatic structures. While the list of species that exhibit thickenings has increased substantially, having been described in four and fourteen different orders of the gymnosperms and angiosperms, respectively (Aleamotu‘a et al. 2019), we remain no closer to understanding the functions that these structures play within the root. Thus, the intent of this review is to focus primarily on the functions of phi thickenings, and as such, discussion of the distribution, structures, and chemical composition of phi thickenings will be limited. Readers interested in these topics are directed to several relevant reviews (de Melo 2011; Fernández-García et al. 2014; Aleamotu‘a et al. 2019). Furthermore, our discussion of the development of these structures, at both a molecular and cellular level, is also limited. Although there is little primary research on development to review, our thinking on this topic is discussed elsewhere (Aleamotu‘a et al. 2019).

1.6 Suggested Roles for Phi Thickenings

Phi thickenings can be induced in roots by a range of abiotic and biotic stresses (Sect. 3). Because of this induction, and because basic structure of phi thickenings has been conserved across diverse taxa from gymnosperms to angiosperms, it is normally assumed that there must be a functional reason for the plant to make these structures. Various functions of phi thickenings in plant roots have been proposed, summarised into three broad themes. Thickenings might:

- provide mechanical strength or support to the root,

- regulate apoplastic transport in a manner analogous to the Casparian strip, and,
- regulate interactions of the root with fungi and other microorganisms.

In this review (Sects. 4–6), we consider the evidence for and against each of these functions. We conclude that phi thickenings are likely to function differently in roots of different species, and that in certain circumstance, all three proposed functions are likely to be valid explanations of phi thickening function. However, we suggest that the default function for phi thickenings is to mechanically strengthen the plant root, and that only in certain cases have phi thickenings been re-tooled to perform other roles.

2 Four Experimental Systems for Testing Phi Thickening Functions

A fundamental problem in considering the function of phi thickenings is the paucity of experimental studies that have been conducted to assess their function. Almost exclusively, data concerning phi thickenings is limited to a description of their localisation in different species or, in some cases, to descriptions of factors that induce their formation. Specific tests of function have, however, been rare. In this section, we define four experimental systems that we have recently used and explain why they provide excellent experimental approaches in which to investigate the functionality of phi thickenings. These model systems differ, however, in the locations in which the phi thickenings develop within the root. The suggested models are:

1. roots of the Geraniaceae, notably in species of *Pelargonium* and *Geranium*,
2. aerial roots of epiphytic orchids, including species from the genera *Laelia, Rhyncholaelia,* and *Cattleya,*
3. roots of the Brassicaceae, notably *Brassica oleracea* and *B. napus*, and.
4. reticulate networks present in the inner face of the inner cortex of roots from the Brassicaceae.

These four systems show significant positional differences in their phi thickenings. In his initial review, Van Tieghem (1888b) noted that there were three different localisations of thickenings within roots. Type I thickenings, as exemplified by the *Brassica* root and the reticulate network, are found in the cell layer immediately outside the endodermis. Type II thickenings are found in roots of species from the Geraniaceae and are localised to the cortical cell layer immediately beneath the epidermis. Type III thickenings are found in the intervening cells of the root cortex, in between the peri-epidermal layer (to use Van Tieghem's terminology) and the sub-epidermal layer, with the *Laelia* root providing an excellent example of this localisation. Thus, these four systems can be thought of as complementary. We include information about these systems in the hope that others might be inspired to use these as models in their own research into the functions of phi thickenings.

2.1 *Roots from* Geranium *and* Pelargonium

Phi thickenings in the Geraniaceae occur in the cell layer immediately below the epidermis, as observed by Bergendal (1883) in *Geranium molle* and *G. sylvaticum*, by Van Tieghem and Monal (1888) in a wide range of species from the genera *Geranium*, *Pelargonium,* and *Erodium*, and by Kroemer (1903) in *G. rotundifolium.* More detailed histochemical and structural investigations were provided in numerous papers between the 1920s and 1980s in which *G. rotundifolium* (Scott and Whitworth 1928), *P. hortum* and *P. peltatum* (Haas et al. 1976; Peterson et al. 1981; Perumalla et al. 1990; Peterson and Cholewa 1998; Meyer and Peterson 2011) were studied. These studies confirmed the network of lignified phi thickenings in the sub-epidermal layer that develop near the root tip and showed that the thickenings were typically semi-circular in shape in individual cells so that the paired structure did indeed appear like the Greek letter phi. The studies also demonstrated that in older parts of the root, smaller thickenings could develop in cell corners in multiple layers of cells deeper into the cortex.

The most significant advantage of *Geranium* and *Pelargonium* roots for testing phi thickenings is their constitutive formation immediately below the epidermis, making for more practical cell biological experimentation than in species where the thickenings are located deeper within the root. This localisation allowed the permeability of phi thickenings to be tested with fluorescent apoplastic tracers, with experiments demonstrating dye diffusion through the root as far as the endodermis, thus questioning the possible role of phi thickenings as an apoplastic barrier (Peterson et al. 1981; Perumalla et al. 1990; Meyer and Peterson 2011). The interpretation of this data will be discussed further (Sects. 5.2 and 5.3). The Geraniaceae system does, however, have one major weakness for studying the function of phi thickenings: with thickening formation being constitutive, and with no known cultivars in which thickenings do not form, and no known inhibitors of the thickening formation pathway, control experiments that lack phi thickenings are not yet possible.

We recently commenced a reinvestigation of the *Geranium* and *Pelargonium* roots. Rather than growing roots from stem cuttings, or taking them from plants growing in soil, we used surface-sterilised seeds of two species, *Geranium robertianum* and *Pelargonium australe*, grown aseptically on agar plates as this provides an experimental system more suitable for the application of drugs and other compounds. Using lignin and cellulose stains (basic fuchsin and calcofluor white) and confocal imaging, we confirmed that phi thickenings form a complex network of lignified walls in the sub-epidermal cells (Fig. 1) beginning only several millimetres from the root tip. Reconstructed root cross sections, generated in ImageJ from high resolution optical stacks, showed that the highly elongate thickenings occupied much of the cells' radial walls (Fig. 1b). Moreover, while the outer face of these sub-epidermal cells was also weakly lignified (Fig. 1b, arrow), extra phi thickenings present in other layers of the cortex were not present. These three observations were made in both species, but run counter to previously published reports. One possible

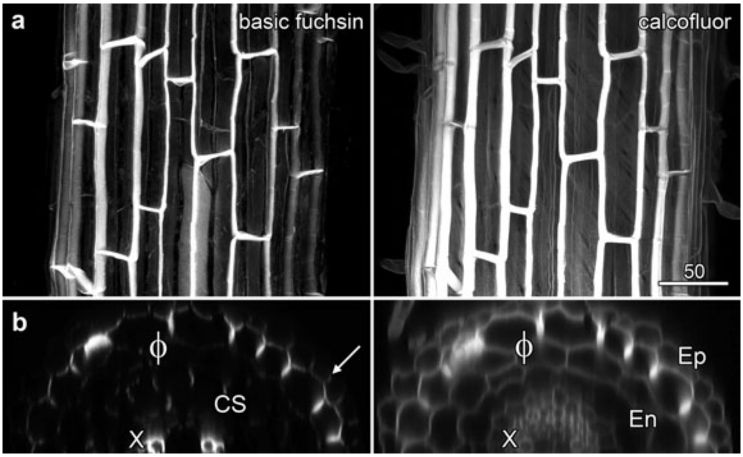

Fig. 1 Confocal imaging of roots of *Pelargonium australe*. Whole roots were fixed, cleared, and then double-labelled with basic fuchsin that stained lignin (left column) and calcofluor white for cellulose (right column). (**a**) Maximum projection of a longitudinal section collected near the root tip showing the sub-epidermal phi thickenings. (**b**) Cross sections generated using the reslice function in ImageJ. These (and subsequent computer-generated cross sections) come from optical stacks collected with the confocal pinhole minimised in order to optimise Z resolution. The weakly lignified Casparian strip (CS) is a broad band within the radial wall of the endodermis. The sub-epidermal cells contain phi thickenings (ϕ) that extend along most of the radial wall, and there is some lignification of the outer wall of this cell layer (arrow). Scale bar = 50 μm for both images. *Ep* epidermis, *ϕ* phi thickenings, *En* endodermis, *CS* Casparian strip, *X* xylem

explanation for these differences is that the narrow primary roots that form following seed germination on agar plates produce slightly different phi thickenings and anatomy to roots induced from cuttings when grown in soil. While the structures that we observed in *Geranium robertianum* and *Pelargonium australe* have some similarities to a hypodermis, the sub-epidermal structure present in the roots of many plant species (Perumalla et al. 1990; Peterson and Perumalla 1990), the strong secondary cell wall indicates that they are phi thickenings.

2.2 *Roots of Epiphytic Orchids*

Our second model system for research into phi thickenings is the epiphytic orchid root and, in particular, the aerial roots from species in the Cattleya group including those from the genera *Cattleya, Laelia* and *Rhyncholaelia*. While phi thickenings are rare in monocots (de Melo 2011; Fernández-García et al. 2014; Aleamotu'a et al. 2019), they are common in orchids, being more frequent in epiphytic and lithophytic species where roots grow in little or no soil, than in species growing in soil (Olatunji and Nengim 1980; Burr and Barthlott 1991). Our initial characterisation of orchid phi thickenings investigated the structure and development of thickenings in the

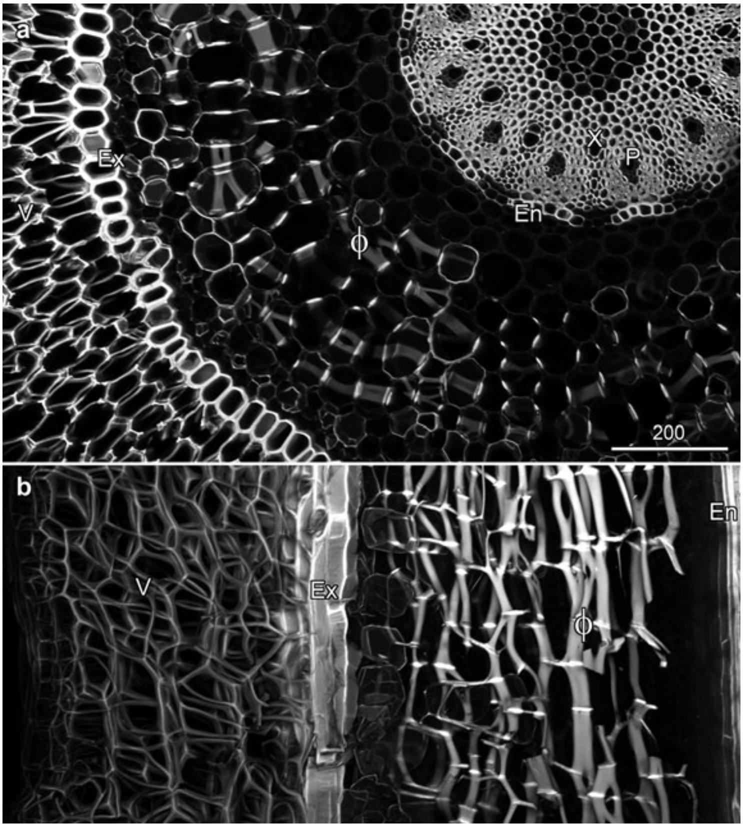

Fig. 2 Confocal imaging of aerial roots of the epiphytic orchid *Rhyncholaelia digbyana*. Images are maximum projections of berberine-stained lignin in sections near the root tip. (**a**) Cross section showing extensive cortical phi thickenings (ϕ). (**b**) Longitudinal section showing the complex organisation of the phi thickening network. The velamen layer (V) that surrounds the root is another complex, lignified network. Scale bar = 200 μm for both images. *V* velamen, *Ex* exodermis, *ϕ* phi thickenings, *En* endodermis, *X* xylem, *P* phloem

South American epiphyte *Miltoniopsis* sp. (Idris and Collings 2015), and we subsequently investigate the function and induction of these structures showing that their presence was unrelated to the development of orchid mycorrhizae, that they did not block the uptake of fluorescent tracer dyes, and that the thickenings were induced by water stress (Idris and Collings 2019). The *Miltoniopsis* root system is not, however, experimentally ideal as the development of thickenings is slow and variable. Instead, we have investigated *Rhyncholaelia digbyana*, a species that has dramatic aerial roots which contain impressive, multiple-ring networks of phi thickenings (Fig. 2). These structures, which in longitudinal sections resemble the reinforcing girders of a building (Fig. 2b), might be a more suitable system for studying the mechanics of phi thickenings. Similar rings of phi thickenings were observed in *Cattleya* roots by Nicolai (1865), and more recently in *Cattleya aurantiaca* (Brundrett et al. 1988), *Cattleya skinneri* (Joca et al. 2017), *Encyclia* sp. and *Prosthechea caetensis* (de Oliviera Pires et al. 2003), and *Laelia anceps* (Idris et al., *manuscript in preparation*).

2.3 Brassica *Roots*

Our third suggested model system for studying phi thickenings is the roots of *Brassica oleracea* and *B. napus*. The initial report of phi thickenings in *Brassica* roots came from an investigation of the cabbage disease clubroot and its causative agent, the bacterial pathogen *Plasmodiophora brassicae* (Woronin 1878):

> Ihre Endodermis oder Schutzscheide nämlich wird umgeben von einer Parenchymschicht, welche durch ihren Bau an die sogenannte secundäre oder Aussenschutzscheide erinnert, die nach den Untersuchungen von Ph. van Tieghem..... den Wurzeln einiger Coniferen zukommt. Diese Eigenthümlichkeit, die bis heutzutage als charakteristisches Merkmal blos für einige Coniferen gilt, ist, wie es aus meinen jetzigen Untersuchungen zu ersehen ist, auch bei den Kohlpflanzen vorhanden.
>
> In den jungen Wurzeln der Kohlpflanzen besitzt, wie bei den Coniferen, jede Zelle der innersten, der Endodermis unmittelbar anliegenden Periblemschicht, an ihren radialen und Querwänden, eine sonderbare continuirliche Membranverdickung; dieselbe ragt in Form einer wulstartigen Ringleiste in's Lumen der Zelle hinein, wobei, was besonders hervorzuheben ist, die sämmtlichen Verdickungswülste aller Zellen dieser Periblemschicht stets einander innigst correspondiren

This text translates as:

> Their endodermis or protective sheath is surrounded by a parenchymal layer, which, due to its construction, is reminiscent of the so-called secondary or outer protective sheath, and which according to studies by Van Tieghem..... occurs in the roots of some conifers. This peculiarity, which until now has been regarded as a characteristic feature of only a few conifers, is, as can be seen from my current investigations, also present in cabbage plants.
>
> In the young roots of the cabbage plants, as in the conifers, every cell of the innermost layer of the cortex immediately adjacent to the endodermis has curious and continuous cell wall thickenings on its radial and transverse walls. These protrude into the lumen of the cell in the form of a series of bead-like bulges, and it should be emphasised, all the thickening bulges of all the cells of this peri-endodermal layer directly correspond.

An extensive investigation of the Brassicaceae by Van Tieghem (1887a) subsequently demonstrated that the phi thickenings are widely distributed in the family, and that their formation varied along the length of the primary root, forming near the tip in some species but considerably further back from the tip in other species. Following Van Tieghem's study, surprisingly little research was conducted on phi thickenings in either *Brassica* or the wider Brassicaceae, a notable omission considering the family's agricultural importance. The first modern images of phi thickenings were provided in a study of the effects of salt on the primary root of *Raphanus sativus* (radish), in which peculiar cell wall thickenings were induced in a cell layer outside the endodermis. Although described as xylem, the published

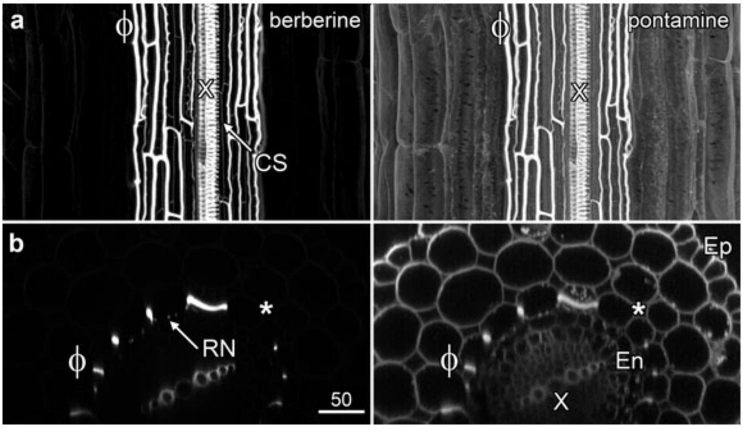

Fig. 3 Confocal imaging of phi thickenings in a commercial cultivar of canola (*Brassica napus*). Roots were fixed, cleared, and then double-labelled for lignin stained with berberine and cellulose with pontamine. (**a**) Maximum projections of a root imaged near the root tip, showing phi thickenings (ϕ) and a faint Casparian strip (CS). (**b**) Computer-generated cross sections show phi thickenings (ϕ) in the inner cortex, immediately outside the endodermis (En). The reticulate network (RN) of lignified thickenings occurs only on the inner face of inner cortical cells. Some cells fail to undergo phi thickening development (*). Scale bar = 50 μm for both images. *Ep* epidermis, *ϕ* phi thickenings, *RN* reticulate network, *En* endodermis, *X* xylem

images demonstrate that the structures are phi thickenings, supplemented by the reticulate network (Sect. 2.4) (Scialabba and Melati 1990). Subsequently, classical phi thickenings were observed in *B. napus* (Enstone et al. 2002), while phi thickenings could be induced in hydroponic cultures of *B. oleracea* (broccoli cultivar Marathon) through the addition of 80 mM salt (López-Pérez et al. 2007; Fernandez-Garcia et al. 2009).

We have used the *Brassica* root as a model system in which to study the induction, development, and function of phi thickenings (Aleamotu‘a et al. 2018). Three-dimensional imaging by confocal microscopy demonstrated that the lignified phi thickenings form a complex cage around the central vascular tissue of the *Brassica* root, and that these structures develop before the onset of suberisation of the endodermis. Confocal imaging of a *Brassica napus* roots double-labelled for lignin and cellulose with berberine and pontamine fast scarlet 4B, respectively, demonstrated the cage-like nature of the phi thickening array (Fig. 3a). Computer-generated reconstructed cross sections through a *B. oleracea* root ~10 mm from the root apex demonstrated three rings of cortical cells, a near complete ring of lignified phi thickenings in the inner cortex, a lignified Casparian strip in the endodermis, and commencement of metaxylem development (Fig. 3b). Intriguingly, in many roots that show extensive development of phi thickenings, individual cells were present in which both cellulose and lignin-labelling demonstrated that thickenings had not been induced (Fig. 3b, asterisk).

In our analyses of phi thickening induction and development, we replaced the hydroponic system of López-Pérez et al. (2007) and Fernandez-Garcia et al. (2009)

with agar plates and have demonstrated that phi thickenings are induced by both salt and sucrose in the primary roots of *B. oleracea* and *B. napus,* and that within these two species, there were distinct variations in phi thickening induction between different cultivars (Aleamotu'a et al. 2018). Moreover, we have recently investigated the mechanics of this induction pathway. Transfer of seedlings from uninduced conditions to inducing conditions causes the formation of a phi thickening network in as little as 24–48 h, and induction can also be triggered by several plant hormones (Aleamotu'a et al.*, manuscripts in preparation*) (Sect. 3).

Multiple advantages exist to study phi thickenings in *Brassica*. Although phi thickenings are common in the Brassicaceae (Van Tieghem 1887a; Aleamotu'a et al. 2018), they are absent from the model species *Arabidopsis thaliana* (arabidopsis) (Aleamotu'a et al. 2018), a species that has only a single layer of cortical cells. While the large collections of mutants and other genetic resources available within arabidopsis cannot be directly used to understand phi thickenings, the close relationship between arabidopsis and *Brassica* means that arabidopsis genomic data might be useful in furthering research into *Brassica* phi thickenings. Several further factors, however, make *Brassica* the best available experimental system for phi thickening research. Several thousand years of selective breeding have produced numerous different cultivars, and the collective genomes of these cultivars, referred to as the pangenome, show high variability. In one study in which nine different *B. oleracea* cultivars were sequenced, not only were more than four million single nucleotide polymorphisms (SNPs) identified, but nearly 20% of the ~60,000 genes identified were entirely absent in one or more of the cultivars (Golicz et al. 2016). More importantly, *B. oleracea* cultivars show wide variability in their ability to induce phi thickenings, with the broccoli Marathon cultivar inducing strongly, and with cultivars such as the kohlrabi Purple Vienna and the cabbage Golden Acre forming few if any thickenings under normal induction conditions (Aleamotu'a et al. 2018). This variability may provide a route to dissecting the molecular pathways that lead to the formation of phi thickenings (Aleamotu'a et al. 2019). *Brassica* roots also have another significant advantage for experiments in which to determine phi thickening functionality. Because thickenings can be induced by specific treatments, including osmotic stress and hormones (Sects. 3.1 and 3.5) (Aleamotu'a et al., *manuscripts in preparation*), roots can be tested under identical conditions in the presence and absence of phi thickening.

2.4 Reticulate Networks in Roots of the Brassicaceae

Roots in the Brassicaceae show an interesting addition to the standard phi thickening pattern, this being the development of a reticulate network that is limited to the inner face of the inner cortical cells (Fig. 3b). We have used lignin staining and confocal microscopy to characterise the three-dimensional organisation of this structure in *Brassica* and other species (Aleamotu'a et al. 2018). In *B. oleracea*, this reticulate network forms as a series of evenly spaced and delicate ridges, less than a

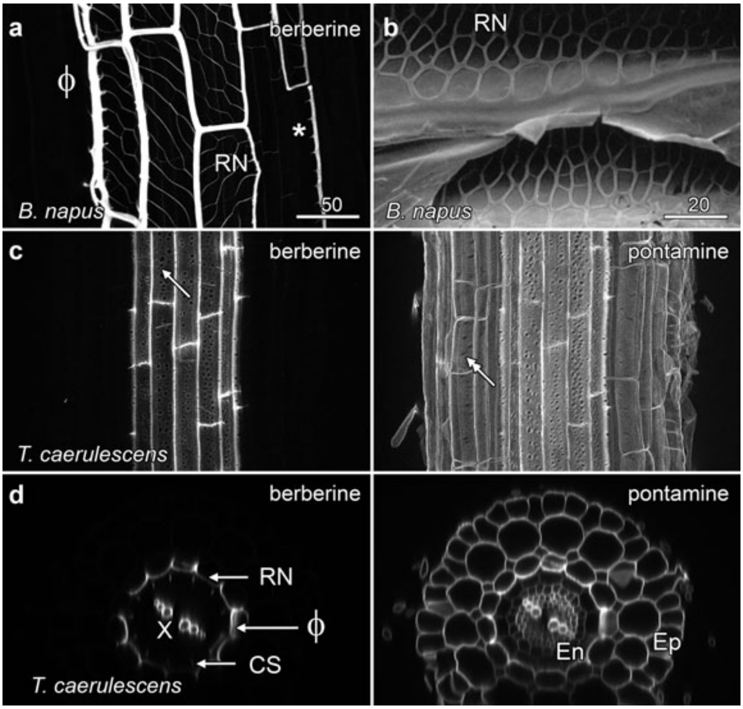

Fig. 4 Confocal imaging of the reticulate network in roots of the Brassicaceae. (**a**) Maximum projection of the lignified reticulate network in *Brassica napus*, stained with berberine. The reticulate network (RN) forms a delicate array on the inner face of the inner cortical cells, linking phi thickenings (ϕ). Several inner cortical cells failed to induce phi thickenings (asterisks). (**b**) SEM image of the reticulate network (RN) in *Brassica napus*. Image courtesy of Dr. Rosemary White and Dr. Margaret McCully, CSIRO Agriculture, Australia, with permission. (**c**, **d**) Confocal imaging of the modified reticulate network in *Thlaspi caerulescens*. Roots were fixed, cleared, and then double-stained for lignin (berberine) and cellulose (pontamine). (**c**) The modified reticulate network is a fenestrated, lignified sheet penetrated by small, round holes (arrow) thought to be pit-fields. Similar elongated pit-fields exist in the primary cell walls of the outer cortical cells (double-headed arrow) but at a lower frequency. (**d**) Computer-generated cross sections of a modified reticulate network (RN) across the entire inner face of the inner cortical cells. Scale bar in **a** = 50 μm for both images except b; scale bar in **b** = 20 μm. *Ep* epidermis, *ϕ* phi thickenings, *RN* reticulate network, *En* endodermis, *X* xylem

micrometre wide and deep (Fig. 4a). Like the phi thickenings, this network initially develops as a cellulosic structure which then becomes lignified. However, while our confocal observations were the first published images of the reticulate network, the original description of this structure was by Woronin (1878) who wrote (in translation) that:

> The characteristic, however, in the structure of these cells is that they are provided on their inner tangential wall with a very fine and delicate net-like thickening. This net originates from the ring of thickenings just described: it grows out of it, so to speak, and then spreads out quite regularly on the inner wall of the cell.

Van Tieghem (1887a) also observed similar structures in a range of different Brassicaceae including *Sinapis alba* (white mustard):

> From each longitudinal band [of the conventional phi thickenings], a series of fine parallel strips extend across the inner, rounded edge of the cell. These bifurcate once or twice, and these branches then unite with branches from the opposite band so as to cover the internal face of the cells with a delicate network. All this network is lignified

Since these original descriptions, the reticulate network has only rarely been discussed in the literature, and in most instances the structural interpretations have been poor. These observations of a reticulate network, however, have always been limited to the Brassicaceae. In a study of salt-induced phi thickenings in radish roots, the reticulate network evident in longitudinal sections was described as xylem that had been induced outside the endodermis (Scialabba and Melati 1990). Similarly, light and electron microscopy images of thin sections of salt-induced phi thickenings in *B. oleracea* roots also identified structures on the inner face of the phi cells that were described as "wall ingrowths" (Fernandez-Garcia et al. 2009). The presence of a reticulate network has also been used to identify mature *Brassica* roots from field samples where wheat and canola had been grown, with scanning electron microscopy images highlighting the three-dimensional nature of the network (*unpublished research*, Margaret McCully and Rosemary White, CSIRO Agriculture, Canberra) (Fig. 4b).

The patterns formed by the reticulate network can vary within and between species. We illustrated subtle differences in the networks formed by *B. oleracea, B. napus,* and *S. alba* (Aleamotu'a et al. 2018), while Van Tieghem (1887a) described four different reticulate network patterns present within the many different genera within the Brassicaceae. We suggest that any attempt to understand the formation and function of phi thickenings also needs to understand the development and function of this Brassicaceae-specific reticulate network. There is, however, a major question to answer here: is the reticulate network specific to the Brassicaceae? As far as we can determine, there has been no work on primary root anatomy and development in other families within the order Brassicales. Based on recent molecular phylogenies, the families most closely related to the Brassicaceae are Cleomaceae and Capparaceae (genera including *Cleome* and *Capparis* (capers), respectively) (Edger et al. 2015). We recently initiated a study to determine whether species in these families contain phi thickenings and to investigate whether the reticulate network is specific to the Brassicaceae.

A role for the reticulate network in nutrient transport has been suggested. In both *Thlaspi* (Zelko et al. 2008) and *Brassica* (Fernández-García et al. 2014), the ridges of the reticulate network have been described as "wall ingrowths" with the resulting increase in plasma membrane surface area suggested to increase nutrient movement. Thus, the reticulate network was being directly compared to the wall ingrowths seen in transfer cells, the specialised cells that form at sites where plasma membrane transport of nutrients is rate limiting, and where highly complex networks of wall ingrowths significantly increase the surface area across which nutrient transport can occur (McCurdy et al. 2008). Three factors argue against this suggestion. First, the amount of extra plasma membrane formed through the presence of the simple and shallow wall ridges in the reticulate network in *Thlaspi* and *Brassica* would only be minimal. Second, the formation of transfer cell-like structures to aid in transport between the inner cortical cells and the apoplast between the inner cortex and endodermis would be a highly unusual location for transfer cells to develop. Not only are these inner cortical and endodermal cells well connected with plasmodesmata, making transfer cell formation redundant, the formation of the Casparian strip blocks *trans*-endodermal apoplastic trafficking. Third, the lignification of the reticulate network would reduce diffusion rates: if these structures were involved in nutrient transport, then lignification of these structures would not be expected to occur. The latter two lines of reasoning will be described in more detail below (Sect. 6.4).

If the standard reticulate network that forms on the inner face of the inner cortex does not normally function in either promoting membrane transport or serve to limit water and nutrient flows, what other functions might it perform? If the structures have any function at all, it is possible that this network may help reinforce the surface of the root as it undergoes secondary growth. During this process, cell divisions initiated in the pericycle, immediately inside the endodermis, are accompanied by the sloughing of the root cortex. This initially leaves the endodermis as the outermost cell layer, and the reticulate network thus forms the outer surface of the root.

Intriguing variations in this standard reticulate network can also occur in species that evolved the capacity to grow in extreme environments. For example, instead of a reticulate network, the heavy metal hyper-accumulator *Thlaspi caerulescens*, also known as *Noccaea caerulescens* (alpine pennycress), forms a lignified sheath across almost the entire inner face of the inner cortical cells that is continuous with the phi thickenings, a structure that has also been referred to as a peri-endodermal thickening (Zelko et al. 2008; Kováč et al. 2020). In an earlier study, this pattern had been described as two layers of endodermis, based on cell wall autofluorescence (van de Mortel et al. 2006), but analysis of the published images, and comparison to the images presented in the computer-generated cross section shown in Fig. 4d, suggests that the first layer of fluorescence to develop represents the inner of two cortical layers in *Thlaspi*, with the second layer developing inside this being the suberised endodermis. This reinterpretation was also recently suggested (Kováč et al. 2020). Similar patterns showing two rings of autofluorescent cell walls also occur in the related hyper-accumulator species *T. goesingense* (Zelko et al. 2008) and *T. montana* (Kutschera and Sobotnik 1992, as cited by Zelko et al. 2008). Furthermore, a similar “double endodermis” was also reported in the extreme halophyte *Thellungiella halophila* (saltwater cress) (Inan et al. 2004) although whether this layer was lignified or not remains unclear. On the basis that the reticulate network localised to the inner face of the inner cortex is widespread in the Brassicaceae, we suggest that the complete lignification of the inner cortical wall in several Brassicaceae species, referred to as peri-endodermal thickenings (Kováč et al. 2020), is simply a modification or elaboration of the reticulate network. As will be discussed in Sect. 6.4, however, this structure may function in the control of ion fluxes within the root.

3 Phi Thickening Induction in Roots

While most plant species do not develop phi thickenings, among the species that do, thickening formation is often subject to regulation by either abiotic or biotic stimuli. It is this induction of thickenings that forms circumstantial evidence that these structures play a role within the root. At least six different types of stimuli can induce thickenings among the different species where they have been identified, including water stress, flooding, biotic stress, heavy metals, hormones, and mechanical stimulation.

3.1 Water Stress

As discussed previously (Sect. 2.3), phi thickenings in the Brassicaceae can be induced by water stress. This effect was initially demonstrated by salt treatment in the roots of both radish (Scialabba and Melati 1990) and broccoli (López-Pérez et al. 2007; Fernandez-Garcia et al. 2009) but we have subsequently demonstrated a similar effect in a range of other Brassicaceae species. More importantly, sucrose also generated this response (Aleamotu‘a et al. 2018) which suggests that the effect is not due to salt toxicity per se, but water stress in general. This conclusion was confirmed recently when the rate of *Brassica oleracea* phi thickening induction was shown to be similar when plotted against the osmotic potential of salt, sucrose, and mannitol solutions, and when induction could be inhibited by the osmoprotectant glycine betaine (Aleamotu‘a et al.*, manuscript in preparation*). Water stress has also been shown to induce the formation of phi thickenings in roots of the orchid *Miltoniopsis* (Idris and Collings 2019). Similarly, in *Eriobotrya japonica* (loquat, family Rosaceae), phi thickenings are also strongly induced in roots by drought stress (Pan et al. 2006).

3.2 Flooding

In contrast to thickenings being induced by water stress, thickenings can also be induced in some species by water-logging. In roots of *Caesalpinia peltophoroides* (sibipiruna, family Fabaceae) phi thickenings develop more strongly when the plants are flooded (Henrique et al. 2009), and so-called crescent thickenings, perhaps a modified form of phi thickenings were more common in *Syzygium samarangense* (wax apple, family Myrtaceae) roots grown in water-logged soils than under control conditions (Tuladhar et al. 2015). Phi thickenings and phi-thickening-like structures have also been demonstrated in numerous aquatic and semi-aquatic plant species associated with the development of aerenchyma, including the mangroves *Rhizophora mangle* (red mangrove, family Rhizophoraceae) (de Menezes 2006;

Souza et al. 2014) and *Avicennia marina* (grey mangrove, family Acanthaceae) (Ashford and Allaway 1995; Allaway et al. 2001) and *Bacopa monnierioides* and *Bacopa salzmannii* (waterhyssops, family Plantaginaceae) (Bona and de Morretes 2003).

3.3 Biotic Stresses

In several cases, phi thickenings have been shown to develop following infection with fungi or oomycetes. According to Melville, *Dryas integrifolia* (mountain avens, family Rosaceae) roots contain few phi thickenings when uninfected, but extensive phi thickenings develop on infection from mycorrhizal fungi (Melville et al. 1987). The question must be asked, however, as to whether these induction events are caused directly by the fungi, rather than a response to the change in the way the root grows following infection. This question will be discussed in more detail below (Sect. 4).

3.4 Heavy Metals

A role for heavy metals in phi thickening induction has been suggested, but the evidence is inconclusive. The heavy metal accumulating species *Thlaspi caerulescens* develops a set of lignified cell walls on the inner face of the inner cortex that would appear to be a modified form of phi thickening and reticulate network (Sect. 2.4) (van de Mortel et al. 2006; Zelko et al. 2008; Kováč et al. 2020), but whether this is actually a response to the heavy metals or a constitutive growth pattern is unclear. While we have demonstrated that *Thlaspi* develops this set of lignified walls without the addition of any heavy metals, and only under water stress (Aleamotu‘a et al. 2018), our experiments have not addressed the question as to whether these cell walls are specifically induced as a response to the presence of toxic heavy metals. Further examination of this question is certainly warranted. Similarly, *Zea mays* (maize) has been reported to form phi thickening-like structures in its rhizodermis when grown in slaggy soils (Degenhardt and Gimmler 2000). However, it remains unclear whether these structures developed in response to heavy metals present, the high salt and pH of the soil, mechanical impedance, or a combination of some or all of these stresses.

3.5 Hormones

The induction of phi thickenings in *B. oleracea* roots is more complex than simply a response to water stress, as thickenings can be induced to different degrees by at least three classes of plant hormones (abscisic acid, gibberellic acid, jasmonic acid) at concentrations that do not overtly reduce the rate of root elongation. In contrast, other hormones, including auxin, cytokinin, ethylene, brassinosteroids, and salicylic acid, did not induce thickenings (Aleamotu‘a et al., *manuscript in preparation*). As far as we are aware, our observations represent the only example of phi thickening induction being shown to be caused by plant hormones.

3.6 Mechanical Stimulation

There are marked effects of growth media on the induction of phi thickenings, with these potentially related to mechanical interactions of the root with the surfaces surrounding them. Thickenings were not induced in *Ceratonia siliqua* (carob, family Fabaceae) roots when grown in perlite, thickenings were common when seedlings were transplanted to soil, an effect suggested to be caused by the mechanical impedance of the soil (Pratikakis et al. 1998). Similarly, *Prunus avium* (cherry) seedlings do not form phi thickenings when grown in agar culture but do so when transplanted to either soil or perlite (Soukup et al. 2004). Furthermore, in *Brassica* roots, a distinct correlation exists between the rate of root growth and the formation of phi thickenings (Aleamotu‘a et al., *manuscript in preparation*). However, maize roots grown in the presence of mechanical impedance did not form phi thickenings (Degenhardt and Gimmler 2000).

4 Biotic Interactions and Phi Thickenings

Links between phi thickening development and the interactions of roots with symbiotic fungi have been proposed. About 90% of plant species form associations with mycorrhizal fungi, and it has been hypothesised that phi thickenings may regulate fungal spread through the root. This hypothesis was developed from analysis of the dawn redwood (*Metasequoia glyptostroboides*) in which phi thickenings first develop near the root tip in cells immediately adjacent to the endodermis. Further from the root tip, more phi thickenings develop in multiple rings in cells further away from the endodermis. Concurrent with this outward development of the phi thickenings, mycorrhizal fungi which initially infect throughout the cortex, except for the cells adjacent to the endodermis, retreat towards the outer cortex. Thus, it is suggested that the phi thickenings prevent the fungus entering the central stele of the root and regulate their presence in the root cortex. This regulation was suggested to happen because the thickenings might help prevent the formation of the intracellular spaces that are important for fungal spread and by limiting the spread of carbohydrates through the cortical apoplast (Böcher 1964). Subsequently, several further studies have made similar conclusions. In *Dryas integrifolia*, few phi thickenings were present in control roots but thickenings were strongly induced by inoculation with the ectomycorrhizal fungus *Hebeloma cylindrosporum* which also caused the distortion of cortical cells (Melville et al. 1987). Similarly, penetration of the fungus *Chloridium* through *Betula alleghaniensis* (yellow birch, family Betulaceae) roots was blocked at the inner cortex by the formation of phi thickenings (Wilcox and Wang 1987). However, functional links between phi thickenings and endomycorrhizal fungi have also been questioned since the 10% of plants that lack mycorrhizal associations include the Brassicaceae, where phi thickenings are common. Furthermore, links between phi thickening formation and the development of mycorrhizal associations could not be detected in either the orchid *Miltoniopsis* (Idris and Collings 2019) nor *Alnus glutinosa* (black alder, family Betulaceae) (Massicotte et al. 1999). However, the absence of an apparent relationship between fungi and phi thickenings in some species does not invalidate interactions in other species.

5 Phi Thickenings: Mechanical Strengthening of the Plant Root

From their initial discovery, it has been assumed that phi thickenings mechanically strengthen the root, with Van Tieghem specifically stating that the "*rectangular frame*" of the phi thickenings would support the cells (Van Tieghem 1871), while a mechanical role for phi thickenings was even more strongly stated by Schwendener (1874) who described the thickenings *"as devices against radial pressure forces"*. In the following 150 years, numerous suggestions have been made regarding a mechanical role for phi thickenings in roots, but no experimental analyses have been published. Indeed, it remains unclear as to what "forces" the phi thickenings might be opposing. In this section, some of the suggestions regarding phi thickenings and mechanical forces will be discussed, followed by a summary of the rather sparse literature suggesting such roles. Finally, we make several suggestions regarding the way phi thickenings may strengthen the root, the role which we propose is the basic function of phi thickenings. In these discussions, however, it is important to remember that lignified walls are not the only contributing factor to the mechanical strength and stability of the plant. Turgor pressure, necessary for cell elongation (Pritchard 1994), is also a major contributing factor to the support of plants, notably in younger tissues where secondary growth has not yet commenced.

5.1 Historical Concepts

Numerous suggestions have been made concerning how phi thickenings might mechanically strengthen roots. Despite Schwenderer's (1874) early suggestion for a role opposing radial forces, most recent suggestions have focused on the longitudinal strength of the root. Having studied *Metasequoia* roots, Böcher (1964) suggested that thickenings should strengthen the root lengthwise. However, having identified that phi thickenings are induced in the apex of carob roots by water stress, it was suggested that the phi thickenings might resist shrinkage of the roots growing in dry soil (Pratikakis et al. 1998), a concept based on Passioura (1988) who listed evidence for the possibility that roots undergo diurnal shrinking under low water potential. In reviewing the phi thickening literature, de Melo (2011) backed this suggestion, but also suggested an alternative whereby phi thickenings might generate a mechanical force that supports cells against the expansion of the central stele. In contrast, Gerrath et al. (2002) suggested that the location of phi thickenings in the region immediately behind the root cap was consistent with a mechanical role in strengthening the root to allow penetration in difficult conditions. It is this positional information with regard to phi thickening formation that we highlight in Sect. 5.3.

5.2 New Concepts

We recently identified two further aspects of root development that support the concept of a mechanical role for phi thickenings. The first example is the observation that phi thickenings are near universal in their orientation, forming bands in a tangential orientation linking the centre of the radial walls. However, the complex patterns of phi thickenings seen in orchid roots provide an exception to this pattern (Burr and Barthlott 1991; Idris and Collings 2015). Of more significance, however, is that some roots with significant aerenchyma can show radially oriented phi thickenings, as seen in mangroves (Ashford and Allaway 1995; Allaway et al. 2001; de Menezes 2006; Souza et al. 2014) and *Bacopa* (Bona and de Morretes 2003). We suggested that this unusual orientation might reflect different forces at play within the roots that contain aerenchyma (Aleamotu'a et al. 2019). Aerenchyma are necessary in these aquatic species to allow for oxygen flow, but these air spaces reduce the mechanical strength of the root. Radially or partially radially oriented thickenings may help the cortex resist the compressive force of water pressure. However, most species that show aerenchyma lack phi thickening-like structures in their root cortex and instead form various cortical rings of reinforced tissue (Striker et al. 2007). These observations demonstrate that there may be different mechanical roles played by phi thickenings in different situations.

The second example is specific to the Brassicaceae where most species typically show a diarch organisation, with only two xylem strands being produced (Bancroft 1930). This means that as protoxylem forms, and then as the metaxylem undergoes exarch development to form a plate of xylem vessels across the root, the root is asymmetrically reinforced. However, the formation of a lignified ring of phi thickenings would provide symmetric reinforcement to the root (Aleamotu'a et al. 2019). While turgor pressure will normally maintain both cell elongation and cell shape within the root, in periods of water stress the presence of phi thickenings may be structurally more important.

5.3 Phi Thickenings as a Mechanical Reinforcement in Plant Roots

The plant root performs multiple functions. Apart from the uptake of water and nutrients, roots are also responsible for anchoring the plant in the ground, meaning that root structure must balance these distinct roles. In considering the mechanical roles that phi thickenings might play within the root, it is necessary to first describe the different forces, tensions, and pressures that act on root tissues.

Young roots and stems show distinct patterns of vascular organisation. Within the stem, peripheral and discrete vascular bundles are composed of xylem and phloem, whereas root vascular tissue is organised into a central stele with alternating xylem and phloem. These differences in vascular patterning are taught as a component of basic plant sciences, but the reason(s) why these fundamentally different organisational states might have evolved are not discussed. These different patterns of organisation are, however, structurally important: in the structural mechanics of reinforced rods, the peripheral placement of reinforcement in the rod results in a larger quadratic moment and provides much higher resistance to flexing (Audoly and Pomeau 2010). Thus, the peripheral positioning of vascular bundles in the stem maximises mechanical strength and allows the stem to withstand the combination of compression, stretching, and torsional forces associated with wind and other disturbances (Fig. 5a). For roots, however, the central positioning of the vascular bundle will provide resistance to stretching forces associated with the wind and disturbances acting on the above ground tissues while minimising root resistance to bending. Thus, the centrally organised vascular tissue of the root makes the root more flexible, allowing it to buckle, and allowing the direction of root growth to be more readily adjusted (Fig. 5b). The centrally positioned vascular system might also provide the opportunity for more filtering of nutrients that are taken up by root hairs and the epidermis and allows for an even distribution of nutrients even if these are collected asymmetrically from the soil.

Young roots and stems undergoing primary growth also show tropistic bending responding to external stimuli such as light, gravity, touch, and so on. This bending occurs through differential cell elongation between the inner and outer sides of the bend, but the locations at which this bending occurs are different in roots and stems. In roots, bending occurs through differential elongation in the distal elongation zone, immediately behind the meristem. In stems that undergo bending, however, the location of the bending can occur one to several internodes below the shoot apical meristem. There are, therefore, important mechanical differences in these two different modes of bending: in stems, the displacement of the site of bending from the shoot apex can mean that bending also moves several leaves, and thus the stem needs considerable reinforcement to allow this to occur. In bending roots, however, the soil would prevent the mass movement of tissue as seen in stems, so bending is necessarily confined to the root apex.

Elongation in stems and roots is also associated with the development of stress asymmetries. If an elongating section of stem is halved lengthwise, the sections bow

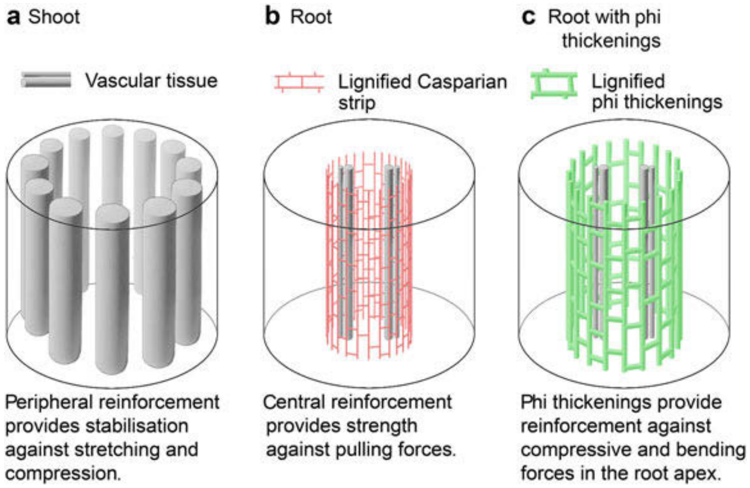

Fig. 5 Stems and roots show different patterns of structural reinforcement related to the way the organs grow and undergo bending. We suggest that the formation of phi thickenings within the root apex functions to make the root mechanically stiffer. (**a**) In shoots, vascular bundles containing fibre cells and lignified xylem occur in the periphery. This reinforcement maximises the quadratic moment providing maximal resistance to bending. (**b**) In the apex of roots, lignified tissue is limited to protoxylem and developing metaxylem in the stele which is centrally positioned and surrounded by the lignified Casparian strip. This location of the vascular tissue minimises resistance to bending, but provides resistance to stretching and compression. (**c**) Many species develop lignified phi thickenings that form a framework around the central stele. For clarity, the lignified Casparian strip is not shown in this image. Our contention is that this network functions to stiffen the apex of the root, a location that is subject to high compressive forces associated with root growth and penetration through the soil

outwards because the epidermal layers, which characteristically contain thicker and often lignified cell walls, are under tension (Kutschera and Niklas 2007). In contrast, when elongating roots are cut lengthwise, the sections bend inwards as it is the central tissues that restrain growth, with the location of the most pronounced bending corresponding to the fastest elongating regions of the root (Pritchard 1994). However, Pritchard suggested, based on unpublished data, that it is not the vascular tissue at the centre of the root that restrains growth, but either the endodermis and/or the inner cortex.

The growing root also experiences forces that are not present in growing stems because root elongation requires penetration through the soil. These forces will cause compression of the root apex and may also result in bending or buckling of the root. Roots navigate a path through the soil directed by gravitational, nutritional, and hydrological stimuli, following a path of least resistance, but root growth is also determined by the mechanical properties of soil that depend on the soil constituents, moisture content, and degree of compaction. The compressive forces generated by elongation in the root tip will be transmitted back along the root, but will be highest in the regions of the root where elongation is occurring. This location is also the site where tropistic root bending will occur, and this is also where xylem reinforcement is limited, and where extensibility of the primary cell walls is highest (Pritchard 1994). Roots typically respond to increased soil hardness through lower growth rates and an increase in root diameter (Atwell 1993). However, several recent studies have investigated the dynamics of root penetration into hard media. For example, when *Medicago truncatula* (barrel medic) roots growing through agar impact a thicker agar substrate, they undergo deformation and buckling in the root elongation and differentiation zones (Silverberg et al. 2012). Similarly, three-dimensional imaging of *Lens culinaris* (lentils) roots showed buckling in root tips in response to compressive forces (Martins et al. 2020), while in *Populus* crosses (poplar), high resolution analysis demonstrated buckling following axial mechanical forces on the root tip (Bizet et al. 2018). In these examples, it is the un-reinforced parts of the root tip that typically show these bending and buckling responses.

The control or limitation of root buckling, and thus the mechanical strengthening of the root, may be an important role for phi thickenings based on the sites in which these structures develop. Multiple studies in different species show that phi thickening development can commence as cells leave the root elongation zone. Among the gymnosperms, this developmental pathway occurs in incense cedar (Wilcox 1962) and *Ginkgo* roots (Bonacorsi and Seago 2016), while in the angiosperms, the same developmental sequence occurs in cherry (Soukup et al. 2004), apple (Mackenzie 1979), and loquat roots (Nii et al. 2004; Pan et al. 2006) from the Rosaceae, carob roots from the Fabaceae (Pratikakis et al. 1998), and in *Geranium* (Scott and Whitworth 1928) and *Brassica* (Aleamotu'a et al. 2018) roots. In these examples, the site of phi thickening formation would be consistent with the secondary cell wall of the phi thickenings being unable to elongate. This location also coincides with, or occurs very soon after, development of both the helically reinforced primary xylem that allows for stretching of the vessel elements and the initial formation of a lignified Casparian strip, but occurs prior to the suberisation of the endodermis. We suggest that the location of phi thickening development in the root apex is significant because it is here that maximum compressive forces develop during root growth, a location where structural reinforcement of the root is limited to the protoxylem.

We propose that the development of phi thickenings in the root apex strengthens the root, and that this is a response in the roots of species where thickenings form in response to stimuli that are linked to difficult and/or stressful growth conditions (Fig. 5c). Quantification of the area of phi thickenings in *Brassica* roots suggests that in the root tip, they can account for considerably more than half of the lignified tissue even after the formation of metaxylem (Aleamotu'a et al. *manuscript in preparation*), so these structures can undoubtedly contribute significantly to root strength. Moreover, the positioning of the thickenings in the root cortex, rather than centrally within the stele as is the case for xylem vessels, would be expected to enhance the structural integrity of the root because their location provides for a large quadratic moment (Audoly and Pomeau 2010). These lignified phi thickenings in the root apex would also limit any contraction of the root due to reduced water potential, an effect that the helically reinforced protoxylem would not be able to counter. Thus, we consider that the likely outcome of phi thickening reinforcement of the root will be a stiffening of the root and a reduction in buckling in the elongation zone and differentiation zone, especially when the root hits obstacles or a more dense substrate. However, as with many aspects of phi thickening biology, a role for phi thickenings in modifying such buckling has not been investigated experimentally.

5.4 Conclusions

In discussing phi thickenings, Schwendener (1874) proposed that they play a mechanical role in strengthening the root, but qualified this statement by saying that it was a hypothesis which he had yet to scientifically investigate. Nearly 150 years later, any mechanical role that phi thickenings play within roots remains unclear. We suggest, however, that the principle role that phi thickenings play within roots is the mechanical reinforcement of the root and that in many cases, this is to prevent or control buckling of the root as it grows through difficult substrates. The evolutionary advantages of a system that enabled better penetration through soils are clear, as this would allow roots, and notably the primary root, to more effectively penetrate soil in adverse circumstances, thus enabling better establishment of young plants.

Furthermore, we note that the induction systems devised in *Brassica oleracea* roots, initially using hydroponics (López-Pérez et al. 2007; Fernandez-Garcia et al. 2009) and more recently simplified with the use of agar plates (Aleamotu'a et al. 2018, Aleamotu'a et al., *manuscripts in preparation*), provide an invaluable tool with which mechanical experiments might be conducted. Not only might the mechanical strength of the whole root be measured, in the presence and absence of phi thickenings, but live imaging penetration experiments analogous to those of Silverberg et al. (2012), Martins et al. (2020) and Bizet et al. (2018) might clarify whether thickenings do contribute to the control of shape in the root apex. Extensive protocols for measuring the strength of plant tissues have also recently been discussed, and although aimed at studying the biomechanics of stems, the concepts and tools might also be directly applied to root tissue (Shah et al. 2017). Similarly, direct measurements of the forces generated by the root tip as it penetrates through a substrate might also be measured using microfluidics. Such experiments have previously been conducted in smaller systems including pollen tubes (Nezhad et al. 2017) and fungal hyphae (Tayagui et al. 2017).

6 Phi Thickenings as an Apoplastic Barrier

Phi thickenings have been suggested to act as an apoplastic barrier because of the similarity in their location and, apparently, their structure when compared to the Casparian strip in the endodermis. This is notably the case for type I phi thickenings in the inner cortex where the thickening-containing cell layer is immediately adjacent to the endodermis. Both the endodermis and inner cortex develop bands of lignification, the Casparian strip and phi thickenings, respectively, that form complete rings around the radial cell walls, and which are aligned from cell-to-cell. This similarity has caused multiple, incorrect identifications in which phi thickenings in the inner cortex have been misinterpreted as the Casparian strip in the endodermis. This erroneous identification was a problem in multiple early publications on apple roots (Nightingale 1935; Stoutemyer 1937; Siegler and Bowman 1939) and remains a problem as seen in the roots of the gymnosperm *Cunninghamia* (Song et al. 2019). These apparent similarities in structure and positioning have meant that phi thickenings have been suggested to regulate solute and water transport into and out of the root's central stele in a manner analogous to the Casparian strip (Mackenzie 1979).

6.1 *The Casparian Strip and the Endodermis*

The formation of a functional endodermis is a multi-stage process, with both the development of a lignified Casparian strip, as well as the deposition of suberin lamellae into the cell wall, being required for a functional endodermis that limits the flow of water and ions into and out of the central stele of the root (Geldner 2013; Doblas et al. 2017) (Fig. 6).

In the young root tip, water and solute movement from the root cortex to the developing stele can occur through either the apoplastic pathway, with diffusion through cell walls (Fig. 6a, red arrows), or through the symplastic pathway where transport occurs from cell-to-cell via plasmodesmata (blue arrows). Endodermal maturation begins with the deposition of the Casparian strip in a complete ring around the endodermal cells (Fig. 6b, green band). This deposition is a tightly coordinated and regulated process, which ensures that the Casparian strips of adjacent cells are fully aligned, and that all cells in the endodermis correctly form the band (Geldner 2013; Doblas et al. 2017). During Casparian strip development, the cell wall at the centre of the radial wall is lignified and the plasma membrane modified so that the membrane remains tightly appressed to the cell wall in a way that blocks the lateral diffusion of fluorescent dyes and proteins through the membrane (Alassimone et al. 2010). The development of the Casparian strip is the first step in controlling the movement of water and solutes from the root cortex into the central stele and limits transport to the symplastic pathway (Fig. 6b, blue arrows). This symplastic transport might, however, occur in one of the several ways. A fully symplastic pathway would be through the collective cytoplasm of the cortical, endodermal, and pericycle cells, all connected via plasmodesmata. Alternatively, plasma membrane pumps and channels might move solutes and water from the endodermal apoplast to endodermal symplast, with other channels and pumps moving material back into the apoplast on the inside of the Casparian strip. This process is sometimes referred to as the coupled trans-cellular pathway (Barberon et al. 2016) (Fig. 6b, magenta arrows). Various channels that localise specifically to

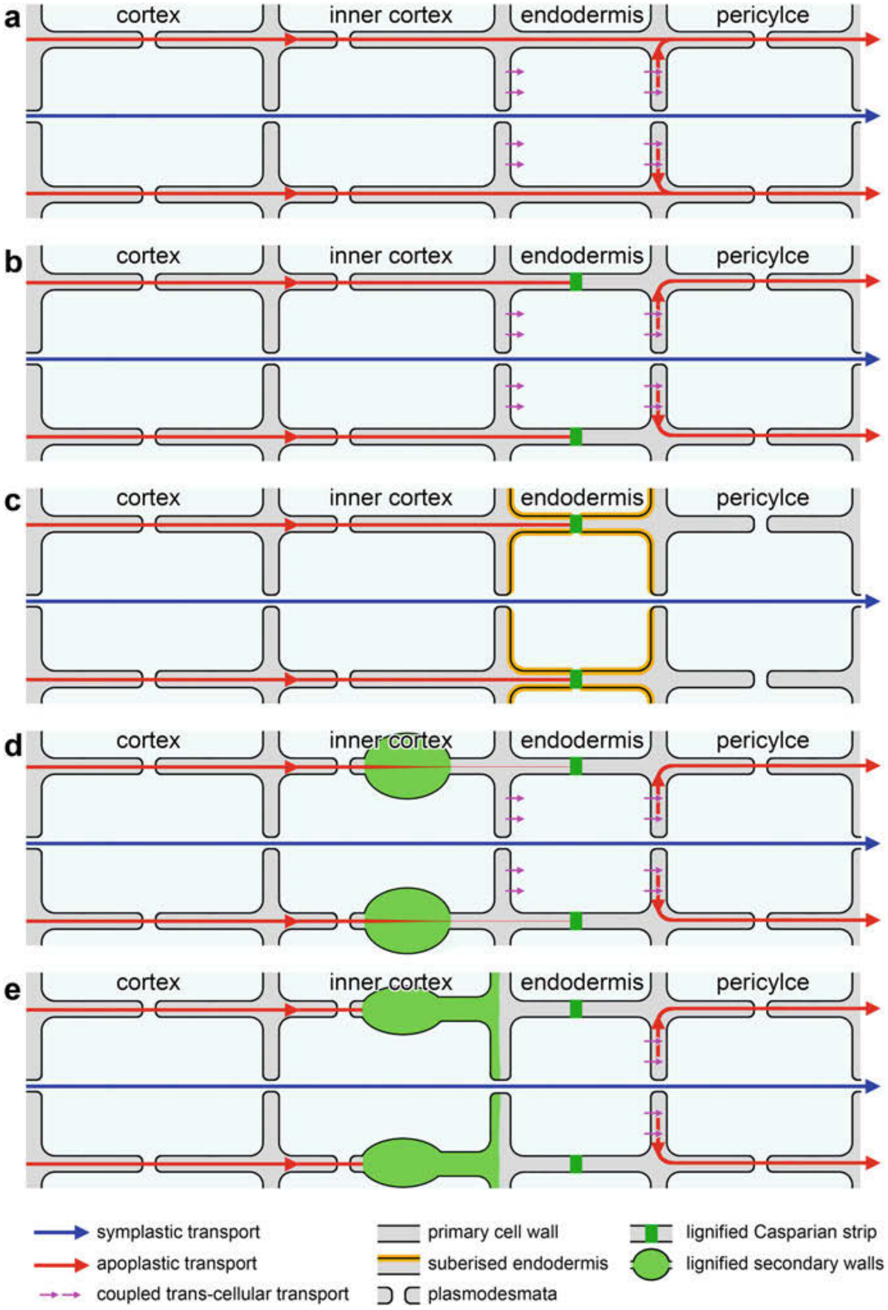

Fig. 6 Apoplastic and symplastic paths for the flow of water and solutes within the root. (**a**) In the very apex of the root, no apoplastic barriers to solute movement have formed, and uptake can be by apoplastic or symplastic pathways. (**b**) Formation of the Casparian strip (dark green) in the first stage of endodermal development prevents apoplastic flows through cell walls to the stele. (**c**) Suberisation (yellow) in the second stage of endodermal development prevents coupled trans-cellular flow across the endodermis. (**d**) Formation of phi thickenings within the root apex of

either the inner or the outer face of the endodermal cells are now known (Bao et al. 2019).

In the second stage of endodermal development (Fig. 6c), layers of suberin, referred to as suberin lamellae, are deposited across the inner face of the entire endodermal cell wall. Suberin, a bio-polyester (Nawrath 2002; Graça 2015), forms a waterproof barrier around the endodermal cell, and reinforcing the block of transport through the wall from cell layer to cell layer provided by the Casparian strip, suberisation will also block transport between the apoplast and symplast (Geldner 2013). Thus, the coupled trans-cellular pathway involving channels and pumps in the endodermal plasma membrane is blocked by this second stage of endodermal development meaning that only trafficking through plasmodesmata will allow water and solutes to pass from the cortex to the pericycle (Fig. 6c, blue arrows).

6.2 Why Phi Thickenings Are Dissimilar to the Casparian Strip

Multiple experiments demonstrate the structural and functional differences between phi thickenings and the Casparian strip and endodermis. First, while both the Casparian strip and phi thickenings are lignified, only phi thickenings show substantial secondary cell wall deposition. Furthermore, the endodermis undergoes subsequent suberisation, a process which does not occur with phi thickenings. This difference has been demonstrated numerous times (Kroemer 1903; Scott and Whitworth 1928; Wilcox 1962; Mackenzie 1979; Peterson et al. 1981; Pratikakis et al. 1998; Fernandez-Garcia et al. 2009; Idris and Collings 2015). Second, there is no evidence for the differentiation of the plasma membrane adjacent to phi thickenings. For example, unlike the Casparian strip which holds tightly to the plasma membrane during plasmolysis (Bonnett 1968; Haas et al. 1976; Alassimone et al. 2010), no membrane domain specialisation occurs during plasmolysis of phi thickening-containing cells (Haas et al. 1976). Similarly, the modified reticulate network present in *Thlaspi caerulescens* does not hold the plasma membrane tightly during plasmolysis in that species (Kováč et al. 2020). Third, the Casparian strip and endodermis are constitutively formed in all vascular plants, forming a complete and unbroken ring around the stele (Geldner 2013), whereas in those plants that do form phi thickenings, thickening development is often incomplete. In maize, the phi

Fig. 6 (continued) some species will mechanically strengthen the root, but would be predicted to reduce solute movement to the endodermis and limit coupled trans-cellular flow. (**e**) In roots of certain Brassicaceae species growing under extreme conditions, the inner face of the inner cortex becomes lignified in the root apex. This lignification likely results in reduced access of water and solutes to channels and pumps in the plasma membrane of the endodermis, resulting in increased control over solute uptake. In some species, the endodermal cells might also undergo secondary wall deposition (not shown) thickenings induced by slaggy soil only occur in discrete patches within the root (Degenhardt and Gimmler 2000), whereas in *Brassica*, where complete rings of phi thickenings can be induced, gaps nonetheless will still often occur where individual cells fail to form thickenings (Figs. 3b and 4a, asterisks) (see also Table 1 in Fernandez-Garcia et al. 2009). These irregular gaps in phi thickening deposition would be sub-optimal for regulation of solute and water movements. Furthermore, while there is a continuous seal between the endodermis of the primary root and the endodermis of lateral roots in apple, gaps form in the phi thickening network at this junction (Weerdenburg and Peterson 1983) and also in the formation of the modified reticulate network in *Thlaspi* (Kováč et al. 2020). Finally, the formation of phi thickenings involves ordered deposition of cellulose within the cell wall, controlled by microtubules (Haas et al. 1976; Mackenzie 1979; Idris and Collings 2015, 2019), whereas there is no known relationship between the formation of the Casparian strip and the cytoskeleton.

The concept that phi thickenings might act as an apoplastic barrier has also been directly tested in numerous studies using fluorescent dyes that cannot pass through the plasma membrane and which, therefore, act as markers for apoplastic trafficking. In apple roots, the cell wall stain tinopal was shown to diffuse through phi thickenings adjacent to the endodermis (Peterson et al. 1981), with similar observations made of sub-epidermal phi thickenings in *Pelargonium* using tinopal (Peterson et al. 1981), calcofluor white (Perumalla et al. 1990), and berberine (Meyer and Peterson 2011). Furthermore, phi thickenings in roots of the orchid *Miltoniopsis* failed to block calcofluor movement (Idris and Collings 2019), while phi thickenings in maize roots did not block berberine flow (Degenhardt and Gimmler 2000). We have also conducted similar experiments showing that the cell wall stain propidium iodide, used elsewhere as an apoplastic tracer (Naseer et al. 2012), passes through phi thickenings in *Brassica* roots (unpublished data). Intriguingly, however, propidium iodide does not penetrate through the modified but heavily lignified reticulate network found in the inner cortex of *Thlaspi* (Kováč et al. 2020).

At first glance, therefore, these experiments suggest that phi thickenings do not act to regulate apoplastic transport, a conclusion we too suggested recently (Idris and Collings 2019). However, exceptions to this conclusion exist. The most direct evidence that phi thickenings can regulate apoplasmic flow comes from *Brassica oleracea* roots, where the uptake of the heavy metal lanthanum, which acts as an apoplastic tracer and can be directly visualised by electron microscopy, was reduced by the presence of salt-induced phi thickenings (Fernandez-Garcia et al. 2009). Furthermore, *Brassica* roots in which phi thickenings had been induced were also found to have reduced hydraulic conductivity and decreased apoplastic flow of water into the roots (López-Pérez et al. 2007).

Can these two different sets of experimental evidence be reconciled? As Peterson et al. (1981) noted in the conclusion to their initial study showing the free access of dye through phi thickenings, their results do not exclude the possibility that phi thickenings reduce rates of water and solute movement through the apoplast. An understanding of the structure of the Casparian strip and a consideration of the evolutionary implications of phi thickening biology suggest that any differences in interpretations derived from these data sets can be resolved.

6.3 An Evolutionary Perspective on Phi Thickenings

Phi thickenings are an ancient evolutionary advance in land plants, conserved in gymnosperms, and found in both the monocots and eudicots in the angiosperms (Aleamotu‘a et al. 2019). Furthermore, a surprisingly detailed fossil exists record for phi thickenings in gymnosperms (Basinger 1981; Millay et al. 1987) with the oldest fossils from the Cordaitales, a sister group to conifers, dating from the late Carboniferous period about 300 million years ago (Lignier 1906; Strullu-Derrien et al. 2009; Césari et al. 2012). Phi thickenings are not, however, known in the ferns. In contrast to this, the endodermis has been suggested to have evolved around 400 million years ago (Geldner 2013) although being in essentially all vascular plants including the ferns, its origins would predate the split between the ferns and the spermatophytes that is now dated to between 420 and 450 million years ago (Morris et al. 2018). Thus, the evolution of the Casparian strip and endodermis predates the development of phi thickenings.

Unlike the Casparian strip and endodermis, phi thickenings are not essential to plants, and most species do not form them. Nevertheless, the retention of phi thickenings within these different taxa implies that they play some important function(s). However, if we assume that phi thickenings are important, then why do only some species contain them? And more interestingly, why in many species are phi thickenings not constitutively present within a root, instead being inducible in response to stress? It is tempting to suggest that if phi thickenings were such an important characteristic to have been conserved during evolution, then all roots should show these structures, just as all roots in land plants retain an endodermis and Casparian strip.

When phi thickenings are considered in this evolutionary context, a simple solution presents itself. The reason that phi thickenings are not constitutively produced in all plant roots is that there is some downside, or negative consequences for roots to form phi thickenings, with the plant having to balance the presumed mechanical advantages of inducing phi thickenings with any disadvantages that their presence causes. We suggest two possible ways in which phi thickenings might be disadvantageous. First, thickened and lignified secondary walls are energetically expensive to produce, as noted previously (Gerrath et al. 2002). A more likely explanation, however, is that phi thickenings act to reduce water and solute uptake. This regulation would be different from that seen with the Casparian strip where the very tight control of nutrient movement forces symplastic uptake of nutrients. Instead, the presence of phi thickenings in the cortical cell walls would slow the diffusion of water and nutrients through the cell wall. While this slowed uptake might be helpful in specific circumstances, for example, in roots growing in extreme environments that are high in salt or toxic metals (Sects. 3.4 and 6.4), the basic function of the root is nutrient and water uptake. Thus, decreases in the diffusion of water and ions through the cortical apoplast would presumably be detrimental. For this concept to be valid, however, one would need to assume that a major pathway for nutrient uptake is through the apoplast, even after the formation of the Casparian strip. However, the coupled trans-cellular apoplastic route for water and solute trafficking into the central stele (Barberon et al. 2016) that requires the presence of specific transporters in the plasma membrane of endodermal cells (Bao et al. 2019) demonstrates this possibility.

The concept that phi thickenings slow the uptake of nutrients is consistent with both physiological data and the anatomy of roots that have phi thickenings. In *Brassica* roots, salt-induced phi thickenings have been reported to reduce hydraulic conductivity of the root (López-Pérez et al. 2007) and uptake of the heavy metal apoplastic tracer lanthanum (Fernandez-Garcia et al. 2009). More importantly, the site of formation of phi thickenings in most species in which their developmental sequence has been investigated is consistent with the physiology of water uptake which occurs predominantly in the root apex, from where protoxylem begins to differentiate through to the differentiation zone where endodermal suberisation commences (see Fig. 5.8 in Kramer 1983). This location is also the region in which phi thickenings are formed in the majority of roots in which the developmental sequence of phi thickening deposition has been identified. As discussed in Sect. 5.3, phi thickenings characteristically develop in the root apex concurrent with or soon after the formation of the Casparian strip and protoxylem, and prior the formation of extensive metaxylem (for example, Wilcox 1962; Mackenzie 1979; Soukup et al. 2004; Bonacorsi and Seago 2016; Aleamotu‘a et al. 2018). After the development of the Casparian strip (stage I of endodermal development, Fig. 6b), uptake of water and nutrients into the root can occur by either the symplastic pathway or the coupled trans-cellular route. If phi thickenings are induced within the root apex prior to suberisation of the endodermis, they will result in a mechanically strengthened root in which the coupled trans-cellular pathway of water and nutrient uptake into the central stele is significantly reduced (Fig. 6d). Thus, the phi thickenings can act in a similar way to other apoplastic barriers such as the Casparian strip and hypodermis that occur within the root, where lignified cells reduce apoplastic flows (Schreiber et al. 1999). We contend that this is not, however, the normal function of the phi thickenings, but a negative consequence of their presence.

6.4 *The Specific Case of Brassicaceae Growing in Extreme Conditions*

In the preceding section, we proposed that any role that phi thickenings might play in the regulation of water and solute uptake occurs solely as a by-product of their role in mechanically strengthening the root. However, as noted in the introduction (Sect. 1.6), specific cases exist where phi thickenings have been re-tooled to play new and different roles within the root. Modifications to the reticulate network in some Brassicaceae species are an example of such re-tooling.

Certain Brassicaceae species that can grow in extreme conditions lignify much of the inner face of the inner cortex. As this cell wall develops in the location where the reticulate network normally develops in the Brassicaceae, we view this structure, sometimes described as a peri-endodermal thickenings, as a modification of the reticulate network. These patterns occur in several heavy metal hyper-accumulating *Thlaspi* species (van de Mortel et al. 2006; Zelko et al. 2008; Aleamotu‘a et al. 2018; Kováč et al. 2020) and the extreme halophile *Thellungiella halophila* (Inan et al. 2004). A role for this modified reticulate network in regulating heavy metal uptake was proposed based on the observation that *Thlaspi arvense*, a close relative of *Thlaspi caerulescens* that does survive on heavy metals, lacks phi thickenings and the modified reticulate network (Zelko et al. 2008), and recent physiological evidence has provided strong support for the concept (Kováč et al. 2020).

The development of modified reticulate network formation in *Thlaspi* roots occurs prior to the suberisation of the endodermis (Kováč et al. 2020). This lignified cell wall would limit flows of solutes through the outer endodermal cell wall and limit movement of material through the endodermis to a solely symplastic route (Fig. 6e). Three pieces of experimental evidence are consistent with this model. First, the apoplastic tracer propidium iodide that passes through phi thickenings in *Brassica* roots (our unpublished data) does not pass through the modified reticulate network in *Thlaspi* (Kováč et al. 2020). Second, energy-dispersive X-ray (EDX) analysis by electron microscopy shows that heavy metal ions accumulate in the cortical layers in *Thlaspi* roots compared to the central stele (Kováč et al. 2020). And third, the high density of pit-fields present within the cell wall linking the inner cortex and the endodermis, compared to the lower density of pit-fields present in outer cortical walls, is also consistent with trafficking reliant solely in a symplastic pathway (Fig. 4c) (Aleamotu‘a et al. 2018).

These observations suggest that the modified reticulate network has a specific role that is not mechanical in nature. Moreover, as many Brassicaceae species have evolved the capacity to grow in toxic environments, undergoing local speciation events in the presence of heavy metals (Baker 1981; Reeves et al. 2018), we predict that one aspect of coping with toxic metals in many of these species will be the modification of the reticulate network in order to reduce solute uptake by the root apex.

These developments would be typical of the strategies that are used to cope with growing in difficult environments. Species growing on soils containing heavy metals are often being grouped as excluders and hyper-accumulators (Baker 1981; Reeves et al. 2018), with the examples of the Brassicaceae in *Thlaspi* described above being hyper-accumulator species in which the heavy metals accumulate in the above ground organs of the plant. The fact that these species can survive even in the presence of high concentrations of otherwise toxic heavy metal ions does not mean that they lack mechanisms that might minimise ion uptake. Many plant species growing in soils containing high levels of toxic metals develop novel root anatomies. For example, phi thickenings and additional cortical lignification occur in the selenium hyper-accumulator *Cardamine hupingshanensis* (Brassicaceae) (Xiang et al. 2019), while in metal-hyper-accumulating genotypes of *Senecio coronatus* (family Asteraceae), novel structures unrelated to phi thickenings develop in the inner cortex but these structures are absent in non-hyper-accumulating genotypes (Mesjasz-Przybyłowicz et al. 2007). Similarly, *Salix* spp. (willow) clones that have higher tolerance to the heavy metal cadmium show increased development of suberised and lignified exodermal and endodermal layers (Lux et al. 2004). In conclusion, the re-tooling of the reticulate network to provide some type of barrier to ion movements through the root tip seems to have occurred in several Brassicaceae species although the precise mechanism through which this might function remains unclear. Nevertheless, we believe that further investigation of phi thickenings and the reticulate network in extremophile species of the Brassicaceae, whether heavy metal excluders of hyper-accumulators, is certainly warranted.

6.5 Conclusions

A role for phi thickenings in regulating water and solute uptake in the manner of the Casparian strip, as previously proposed (Mackenzie 1979), seems to be a highly unlikely function for phi thickenings. Instead, we suggest that phi thickenings might reduce water and solute uptake as a by-product of their presence within the root, and that this downregulation is balanced against the mechanical strengthening that the thickenings provide to the root. In specific case, however, phi thickenings, or more specifically the reticulate network associated with the phi thickenings in the Brassicaceae, may have evolved into a distinct, lignified barrier that acts in conjunction with the Casparian strip to provide filtering of solutes.

7 Future Directions

In the 150 years since phi thickenings were first illustrated (Van Tieghem 1871), and despite the structures having been identified in a wide range of species, the role(s) of these enigmatic structures within the root remains unclear. In this review, we suggest that the primary role of the phi thickenings is mechanical, but that these structures have most likely been re-tooled by evolution to perform different roles in different species.

To date, the study of phi thickenings has been the preserve of plant anatomists and, occasionally, plant physiologists, cataloguing species in which they are found, and identifying stimuli that might cause their formation. The future for understanding phi thickening functions, however, will lie with modern genetic and interdisciplinary science, and to this end we have identified four systems that we consider the most suitable for investigating functionality. The genetic diversity present within *Brassica oleracea* and *B. napus* populations and the ability to reliably trigger phi thickening formation through hormonal treatments suggest that the developmental pathway through which thickenings form might be readily identified. We have previously discussed the concept of an as yet unidentified master regulator for phi thickening development, a transcription factor(s) that turns on the formation of the multiple cell wall formation pathways required to make functional, spatially coordinated phi thickenings (Aleamotu'a et al. 2019). Similar master regulators exist for other secondary cell walls such as xylem development (Oda et al. 2010) and pathways regulating development of other novel cell walls such as transfer cells have been identified (Nguyen et al. 2017; Offler and Patrick 2020). The identification of similar regulators and pathways of phi thickening development through genetic approaches will be important, and technologies such as single cell sequencing (Denyer et al. 2019; Jean-Baptist et al. 2019), proteomics (Petricka et al. 2012), and genome wide association studies (GWAS), already routinely conducted in *B. napus* populations (Gacek et al. 2017; Raman et al. 2019), may all prove of value. Meanwhile, multiple different mechanical strength tests might be performed on *Brassica* roots with induced thickenings (Shah et al. 2017), and these results might be checked by mathematically modelling the phi thickening cell walls (Geitmann and Ortega 2009, Bidhendi and Geitmann 2018).

Considering the limited body of research investigating phi thickenings over the last 150 years, are these structures of sufficient significance to warrant further investigations in light of the many other challenges facing plant sciences? We argue that if phi thickenings do help stiffen the root tips of species from angiosperms and gymnosperms, then this attribute must be significant. Moreover, the induction of these structures in response to various stresses also argues for their functional importance. A mechanism enabling enhanced penetration of roots into soil, particularly in adverse conditions, thus facilitating access to water and nutrients to support plant growth (Padilla and Pugnaire 2007), clearly has considerable importance to agriculture.

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