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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. All chapters are thoroughly peer-reviewed by at least two independent referees.

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Part I
Review

Ants, Plants and Fungi: A View on Some Patterns of Interaction and Diversity

Andreas Bresinsky

Contents

Abstract Biodiversity is a challenging field of research. Approaches are manifold and mostly cover few aspects of the total wealth of phenomena only. The mapping of the vascular plants and the inventory of Basidiomycota in Bavaria are projects to be mentioned in this context as one part of the commitment of the author. In the following article the author describes further activities during his lifework in regard

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to ant–plant interactions resulting in dispersal and distribution of plants, and moreover, in respect to speciation and to evolution of high ranked taxa within the fungi, discussed mainly in relation to the genus *Pleurotus* and the order Boletales. In fungi the investigations include breeding systems, isolation barriers, polyploidy, pigment patterns and DNA phylogeny. The pigment patterning in Boletales correlates well with the phylogeny as revealed by DNA analysis of selected gene sections.

1 Introduction

It is a great honour for me to be invited by the editor of this book series, "Progress in Botany", to discuss the results of studies which came about following my scientific interests in the review of my life. My doctoral thesis on the dispersal of seeds and fruits by ants had been published 50 years ago (Bresinsky 1963). Taking that study as a kind of my personal starting point, I intend to focus on rather different scientific topics on which my research work was directed. An arch has to be spanned in order to link such different organisms as ants, plants and fungi. Ecological interactions between these groups of organisms would basically allow to develop a consistent presentation. However, actually it is impossible for me to report on my work without taking into account some frictions in the context. Looking back on my life as a scientist, dealing mostly with plants and fungi, I regret that my work had not been concentrated in a more stringent manner on a single integrative topic including all of the mentioned organismic groups.

Being able to perform my life in the world of science came true by the guidance and efforts of my deceased teachers: Dr. Hans Doppelbaur and Fritz Beinroth in the period when I was going to school in Augsburg, and the professors Karl Mägdefrau, Hermann Merxmüller and Josef Poelt in the time when I was visiting the University at Munich. I am grateful that an enriching personal friendship from which I draw permanent benefit until now developed to all of them over the time. After my graduation from the Ludwig-Maximilians-University in Munich I started with my professional career as an employee of well-known institutions, first at the Technical University (TH under Prof. Dr. O. Kandler) and then at the Bavarian State Collection of Botany (Botanische Staatssammlung under Prof. Dr. H. Merxmüller), both in Munich. A really lucky turn in my life was my appointment as full professor and head of a research unit (Lehrstuhl) at the newly established University of Regensburg where I spent the greatest part of my professional life. A highlight during this time in Regensburg certainly was the delegation to the task of convening the International Mycological Congress which had been attended by 1,600 participants from 60 different nations (Hawksworth 1991).

2 Seed Dispersal by Ants

The tremendous biodiversity on the globe has to be studied in respect to historical–phylogenetical developments (evolution) on one side, and in respect to ecological adaptations on the other side. Both modes of interpretation in regard to biodiversity have their own justification and their own importance. A debate about the status and the dominant role of either one of both approaches makes no sense.

2.1 The Challenge of Biodiversity

In more recent studies on biodiversity it is the integration of both approaches allowing to achieve a consistent view. I was much impressed by the field trips offered more than 60 years ago by my academic teacher Karl Mägdefrau, because he introduced the students to the diversity of plants not simply by telling their names and their position within the classification but rather by discussing different groups of plants according to their ecological interrelationships. That was quite appealing in respect to a didactic and scientific approach as well. The way Karl Mägdefrau dealt with the ecological background of biodiversity was not an experimental one, it was not one to measure and analyse environmental parameters, but it was much more directed by the interpretation of features of external and internal structure in respect to the challenge to which plants are confronted with in their different environments. In this sense he was obviously not a representative of an analytically and experimentally based ecology which had become more and more important since a while. His attitude to deal with ecology in a mostly non-experimental mode was clearly connected with his main scientific interest in plant life of the earlier geological periods. In this area of research he was well known in his time, and it is obvious that, in this case, he could achieve approaches in the ecological understanding of biodiversity only through interpretation of correlative interrelationships between structures and environmental conditions as far as these are explorable at present. His standard work on the palaeobiology of plants (Mägdefrau 1935; Paläobiologie der Pflanzen 1. ed.) offers a number of examples for such a kind of approach. It might have guided him in choosing topics to be investigated in doctoral theses.

2.2 Scientific Approach to Myrmecochory

Thanks to the suggestion of Karl Mägdefrau the task had been given to me to deal with an interesting subject which is still in the focus of recent research (Mayer et al. 2005; Peternelli et al. 2008; Pfeiffer et al. 2010; Fokuhl 2008; Fokuhl et al. 2007, 2012; Boiero et al. 2012): studies on the phenomenon of ant-mediated dispersal of

seeds and fruits with special regard to elaiosomes. My decision to deal with this subject had been debated somewhat critically within the then existing community of supervising and learning persons at the Institute of Botany ("Nymphenburg") of the University in Munich. You got to hear controversial statements as for instance "senseless study of more or less meaningless structures (i.e. the elaiosomes causing the spreading of seeds by ants) without ecological importance for dispersal". From today's viewpoint I have to appreciate the critical discussion of those days because the communicated arguments prevented me to be fixed to a given attitude towards the subject I had to investigate.

Actually, many of the diverse interactions between ants and plants give us the impression of mutualistic interrelationships. One example of these interactions is the uptake, transport and dispersal of seeds and fruits if they are equipped with nutrient-rich appendages, the so-called elaiosomes (Fig. 1). Classical investigations on this topic have been accomplished by Sernander (1906). Rutger Sernander (1866–1944) was able to demonstrate that elaiosome-equipped dispersal units (diaspores) are regularly dispersed by ants. In the course of the diaspore-directed activity of ants, it is only the elaiosome which will be damaged or eaten while the seeds or fruits remain intact. As a matter of fact, the publication of Sernander first dealing with this phenomenon has to be dated back some years earlier when he was coining the broader designed term synzoochory (Sernander 1901) which includes among others the dispersal of diaspores by ants. He made lipids (oil), which are regularly present in the elaiosomes, responsible for the transportation of diaspores by ants. According to Sernander diaspores with detached elaiosomes are mostly less attractive for ants; it was also him (Sernander 1927, according to Wagenitz 2003) who created the term diaspore comprising all kinds of dispersal units of plants regardless their structure and developmental origin (homology). Moreover, he described the basic features of a syndrome characteristic for plants dispersing their diaspores by the aid of ants. In regard to its critical and careful attitude the monograph of Sernander (1906) has to be seen as a distinguished example of a study on a special mode of dispersal (of plants by ants; myrmecochory). Within the history of botany by Mägdefrau (1992) the study has found an honourable position in the chapter "plants in their environment".

Worldwide there are more than 3,000 species of herbs, shrubs and trees with diaspores being equipped with structures to ensure dispersal by the aid of ants (Beattie and Hughes 2002). In a more recent survey the number of myrmecochorous species worldwide is roughly estimated to be 11,000 in 334 genera and 77 families (Lengyel et al. 2010). In Central Europe the share of plants out of the total number of species (in Angiosperms, seed plants) being equipped with structures for ant dispersal is comparatively low. However, if the share is related to special units of vegetation or to some of the phenological periods then it might be quite considerable (Ellenberg 1996, for plant species of beech forests flowering in spring time). In the frondose woods (Querco-Fagetea excl. Prunetalia and Alno-Ulmion) of Bavaria the share of myrmecochorous representatives out of all 341 species is 14.8 % and even increases to 18.4 % in the herbaceous and grass layer (Bäumler 1984). In the summergreen deciduous forests of Europe and in those of Northeast America

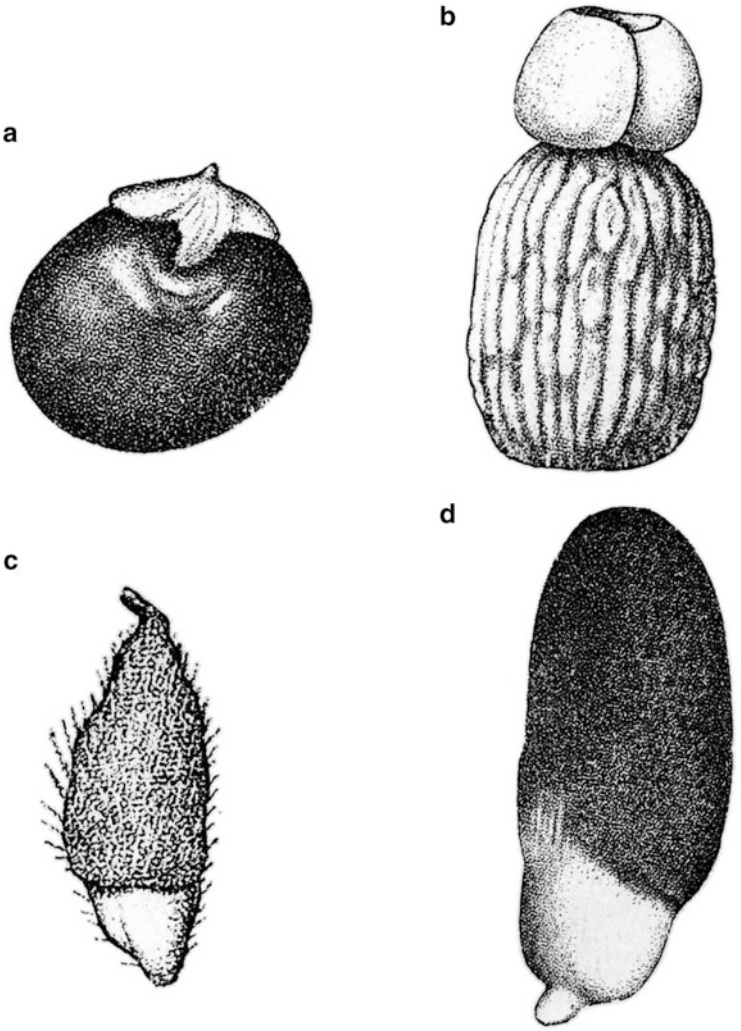

Fig. 1 Diaspores (seeds **a**–**b** or fruits **c**–**d**) with attached elaiosomes. (**a**) *Moehringia trinervia* (see also Fig. 2a). (**b**) *Euphorbia myrsinites* (see also Fig. 2b). (**c**) *Hepatica nobilis* (see also Fig. 2c). (**d**) *Melampyrum cristatum* (see also Fig. 2e). After Sernander 1906 in Morton 1912 (Fig. **c**), Morton 1912 (Fig. **a**, **b**, **d**)

myrmecochory (up to 30 % of the species flowering in spring; Lanza et al. 1992) is mainly restricted on the ground vegetation (Sernander 1906; Kusmenoglu et al. 1989). Myrmecochory was also found to be a device of dispersal in the shrubland vegetation of South Africa (in 20 % of the species) and of Australia (Bond and Slingsby 1983; Berg 1975). Elaiosomes are adapted to the prevailing environmental conditions. The elaiosomes from plants of temperate woods are soft and usually decay in a rather short time if not eaten by ants whereas those from arid areas are

tough, resistant and may persist for a longer time (Beattie 1985). Only few reports are published on myrmecochory in the tropics. There, different from the temperate regions, myrmecochory is often bound to the life form of trees (phanerophytes). In a tropical research area 21 different species of ants in nine different genera ranging in size from 0.1 to 1.2 cm were observed to interact with elaiosome-equipped seeds (Horvitz and Beattie 1980). Also leaf-cutting ants like *Atta sexdens rubropilosa* are active in seed dispersal there (for instance in case of the plant species *Mabea*; Peternelli et al. 2008).

2.3 Ontogeny, Anatomy and Histochemistry of Elaiosomes

At the time when I began my investigations, the anatomy and histochemistry of the appendages on seeds and fruits (diaspores) mediating myrmecochory were only insufficiently explored. The reason for it was the fact that the required sections through the hard outer layers of the diaspores on one side and through the adherent soft appendages with big and thin-walled cells on the other side were not easily to be accomplished. Using a special cutting technique (freezing microtome, embedding of the material to be cut in frozen gelatine) it was possible to obtain reasonable sections (Fig. 2). The development (ontogeny) of the elaiosomes was investigated by using regular microtome techniques. The formation of elaiosomes as myrmeco-attractive structures from quite different parts of seeds, fruits and even inflorescences (as it is also realised in case of the devices to ensure dispersal by wind, anemochory) clearly favours the idea of a strong selective pressure behind it in order to ensure dispersal (Bresinsky 1963). However, at the same time there is also a selective pressure effective to avoid damages to diaspores caused by ants. Taking this into account then the myrmeco-attractive appendages on diaspores could even be interpreted as devices to distract ants from the essential parts of diaspores in regard to their reproductive function. This argument is weakened by the fact that diaspores with removed elaiosomes are mostly unattractive for ants.

Myrmeco-attractive appendages on seeds can arise from the outermost layers of the exterior integument (the so-called sarcotesta of *Puschkinia*, *Ornithogalum*). In case of *Puschkinia* the myrmeco-attractive cells of the integument have the shape of concave watch glasses. Their longitudinal extension is ten times that of the cells in the layer underneath. At the end of their hypertrophic development the cell walls are Sudan positive indicating the presence of a triglyceride. A more advanced localisation and morphology of elaiosomes on seeds is accomplished in different ways: through reorganisation of the outer integument beneath the micropylar region in case of *Scilla*, *Chionodoxa*, *Euphorbia* and *Polygala*, of the chalaza region (opposite to the micropyle) in case of *Galanthus* and *Luzula*, of the raphe (which is a strand of a vascular bundle adnate to anatropous ovules) in case of *Helleborus*, *Asarum*, *Chelidonium*, *Stylophora*, *Corydalis*, *Reseda* and *Viola* pr. p., of the funiculus (a caudicle-like structure bearing the ovule) in case of *Moehringia*, *Claytonia*, *Primula* and *Sarothamnus*. The histological rearrangements in the

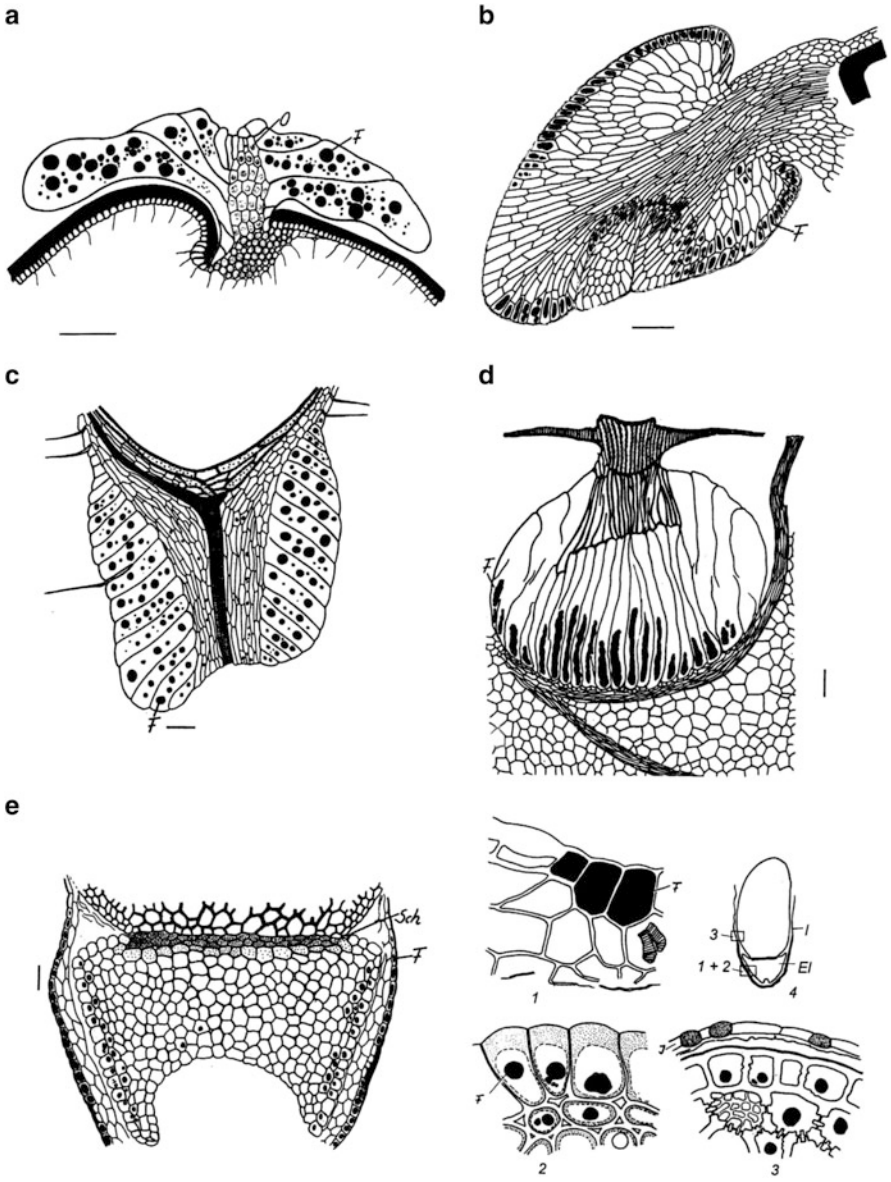

Fig. 2 Anatomical structures of elaiosomes and of adjacent tissues at the diaspores. F = oil (fat), O = oxalate crystal. Bar equals 100 µm. (**a**) *Moehringia trinervia*. (**b**) *Euphorbia amygdaloides*. (**c**) *Hepatica nobilis*. (**d**) *Pulmonaria* spec., top diaspore with elaiosome, below fruit axis with vascular bundle serving as abscission layer. (**e**) *Melampyrum pratense*. *Left side*: The endosperm is divided into an upper part for the nutrition of the embryo and into a lower part with the function of an elaiosome, between both parts an abscission layer (Sch) with introsusception of tannins (*black*). The outer loose envelope is formed by the integument. *Right side*: (1) outer cells of the integument, (2) outer cells of the elaiosome, (3) outer cells of seed above the abscission layer and (4) seed with elaiosome (El) and integument (I). (1) + (2) and (3) = location of the thin sections in the diagrams (1)–(3) (Bresinsky 1963)

mentioned parts of the ovules mostly include a higher rate of cell division, hypotrophic growth of cells and accumulation of special compounds (lipids, etc.).

A special mode of forming the elaiosome could for the first time be described in case of *Melampyrum* (Figs. 1 and 2; Bresinsky 1963). It throws a particular light on the functional interpretation of elaiosomes. Performing no other important function, it is simply unthinkable that a part of the nutritional tissue of the seed (the endosperm) is detached and thus being lost as nutritional potential for supporting the embryo; hereby the primary function of the endosperm (nutrition of the embryo) is considerably reduced. The endosperm of seed plants arises in the course of a double fertilisation. So it becomes a triploid tissue in two different steps, first through fusion of two nuclei in the primordial ovule and then through fertilisation of the diploid nucleus with a haploid nucleus from the pollen tube. In case of *Melampyrum* about one-third of the total endosperm is pinched off and separated from the remaining endosperm by an abscission layer, in a way that damage to the endosperm serving for the nutrition of the embryo is avoided. The separation into two different functional parts would not make sense in regard to dispersal, if the endosperm were covered and protected by a firm seed coat. In *Melampyrum* such a seed coat does not exist, because the integument from which a seed coat regularly arises is only developed to a thin and loose membrane partly enveloping the seed and which may be easily removed from the seed. It is also the integument cap that stores lipids in its outer cells (Fig. 2e). This integument cap is composed of three cell layers in the area of the elaiosome. It becomes much thinner towards the part where the embryo is embedded or even is missing on the top of the seed. The decisive parts of the seed (i.e. the embryo and its surrounding endosperm) are not protected by an integument-borne seed coat but rather by thick-walled cells in the outer layer of the endosperm and further through a tannin-containing abscission layer between the elaiosome and the rest of the seed. In contrast to the main part of the seed serving the nutrition of the embryo the outer cells of the elaiosome are thin-walled and include myrmeco-attractive oil. The findings support the idea that all these structures depend on a selective pressure favouring the dispersal of the seeds by ants and protecting at the same time the embryo and its nutritional tissue. The mimicry theory (ants take and carry the seeds of *Melampyrum* because of some kind of imitation of their larvae or pupae) will be discussed later.

Finally the cases of elaiosome bearing fruits have to be mentioned. In these cases elaiosomes are formed from the exocarp (*Ballota*, *Lamium*, *Anemone*, *Hepatica*, *Ranunculus ficaria*, *Adonis*, *Fedia*), from the receptacle (*Symphytum*, *Pulmonaria*, *Rosmarinus*, *Ajuga*), from the axis bearing the developing fruit (*Aremonia*, *Potentilla* Sect. *Fragariastrum*, *Thesium*), from the persistent basal part of the style (*Carduus nutans*), from the perianth or perigone (*Parietaria*, *Danthonia decumbens*, *Knautia*, *Centaurea* pr. p., e.g. *Centaurea montana*), or from parts of the inflorescence with several reduced flowers, e.g. from reduced parts of spikelets of grasses (*Melica* pr. p., e.g. *M. nutans* with four reduced flowers integrated in the elaiosome; *M. uniflora*).

The assignment of elaiosome development to a special primordial part of seeds and fruits is sometimes not distinctly feasible because the origin may be somewhat

ambiguous. Different parts of the ovule contribute e.g. to the elaiosome of *Viola* (the raphe and the funiculus). The origin may also be uncertain or only deducible from general viewpoints of development, e.g. in case of *Centaurea* pr. p. or *Knautia*: it might be debated whether the elaiosome develops from the envelope or from the axis of the flower. Because of the same reasons of ambiguity the typification of myrmecochory by Sernander (1906), based on function and morphology, has some weak points.

In floral biology the term syndrome is used for the consonance of several features directed to special pollinators. Also in case of myrmecochory it is possible to sum up several properties as a syndrome favouring or enabling the dispersal of diaspores. These are morphological and chemical features. Morphologically the myrmecochory syndrome is characterised by often colourless, juicy-soft, frequently rather voluminous appendages (elaiosomes) as a part of the diaspores. The cells of the elaiosomes are often hypertrophic (Fig. 2a) and contain lipids and other ant-attractive compounds. Sometimes the lipids are stored in the exterior cell walls or cells of the elaiosomes (Fig. 2b, d, e). Quite commonly special feeding barriers against entry into the essential parts of diaspores are established between the elaiosome and the seed or fruit, like tannin-containing cells (Fig. 2e), thick-walled cells or oxalate crystals (Fig. 2a). The exposition of diaspores close to the ground is also a part of the myrmecochory syndrome in temperate frondose woods (Sernander 1906). Not all of the above-mentioned features have to be present simultaneously in order to ensure myrmecochorous dispersal. However, in Central Europe the combination of many or even all of these features are linked with myrmecochory.

In the Mediterranean in evergreen frondose woods and in shrub communities or in Atlantic heath lands some shrub species with a dissemination of diaspores comparatively high above ground (*Rosmarinus*, *Sarothamnus*, *Ulex*) are myrmecochorous, where at first the diaspores may be set free by a ballistic mechanism. Also in Central Europe not all of the myrmecochorous plant species expose their diaspores directly on the ground. Quite a number of species disperse their diaspores by means of a ballistic mechanism or some other device in a wider area around the mother plant, and are then carried off by ants (dichory: some species of *Viola* pr. p., *Euphorbia*, *Lamium*, etc.). Some plants realise a combination of wind dispersal (anemochory) and myrmecochory whereby, however, those structures enabling wind dispersal seem to be reduced (*Centaurea cyanus*) or less effective (*Melica nutans*). A combination of endozoochory and myrmecochorous synzoochory is given in the grass species *Danthonia decumbens*: the plant gets dispersed when the diaspores are passing the digestive tract of cattle (endozoochory) or if they are picked up by ants (synzoochory) because of the myrmeco-attractive tissue on the palea at the ripening stage of flowers (Sernander 1906).

2.4 *Myrmeco-Attractive Compounds in Elaiosomes*

Orientation in the environment and social behaviour of ants are regulated by chemical signals (Wilson 1965; Hölldobler 1978; Bradshaw and Howse 1984; Morgan 2008). In regard to the chemical basis of myrmecochory one has to distinguish between stimulants, attractants and nutrients. The orientation of ants in their environment can be optionally olfactory or gustatory. The stimulants, including those emitted from elaiosomes, are perceived by antennation and answered by some kind of a special reaction. The mode of perception is regarded to be mainly gustatory through direct contact of the antennae with the source of stimulation and less important, if at all, by olfaction of volatile compounds via the air in some distance from the elaiosome (Sheridan et al. 1996). The ants are not attracted by visual cues (Bond and Slingsby 1983; Gammans et al. 2006). The myrmeco-attractive compounds regularly occurring in elaiosomes are lipids (Sernander 1906; Bresinsky 1963) and sugars (Bresinsky 1963). These compounds can be interpreted as attractants and at the same time as nutrients. Moreover, compounds like proteins and vitamins have been detected in elaiosomes (Bresinsky 1963); however, because of sporadic occurrence their function may be not so decisive as compared to lipids and sugars.

2.4.1 Lipids

Lipids are stored in elaiosomes in the form of triglycerides (Sernander 1906; Bresinsky 1963; Kusmenoglu et al. 1989; Lanza et al. 1992; Boulay et al. 2006), diglycerides (Marshall et al. 1979; Skidmore and Heithaus 1988; Kusmenoglu et al. 1989; Lanza et al. 1992; Boulay et al. 2006; Peternelli et al. 2008) and as free fatty acids (Bresinsky 1963; Skidmore and Heithaus 1988; Lanza et al. 1992; Gammans et al. 2005; Boulay et al. 2006; Fischer et al. 2008; Peternelli et al. 2008; Boieiro et al. 2012).

The histochemical proof of triglycerides was done by staining with Sudan III (Sernander 1906; Bresinsky 1963). The use of Sudan III has the advantage that the localisation of the lipids can be microscopically specified in the cells of a tissue and the disadvantage of some degree of non-specificity (e.g. also waxes eventually react). In the case of a missing reaction with Sudan III the lipids may be present in form of diglycerides or as free fatty acids. Actually a proof of the occurrence of a free fatty acid (identified as ricinoleic acid; Fig. 3) could be provided in elaiosomes which did not show any reaction with Sudan III (Bresinsky 1963). In more recent studies advanced separation techniques for identification of lipids have been applied such as thin layer chromatography, column chromatography (Marshall et al. 1979) and gas chromatography (Bresinsky 1963). Using the latter method it became possible to increase considerably the number of free fatty acids being detected in elaiosomes (Skidmore and Heithaus 1988; Peternelli et al. 2008; Pfeiffer et al. 2010). However, the presence of the unsaturated hydroxy fatty acid, ricinoleic

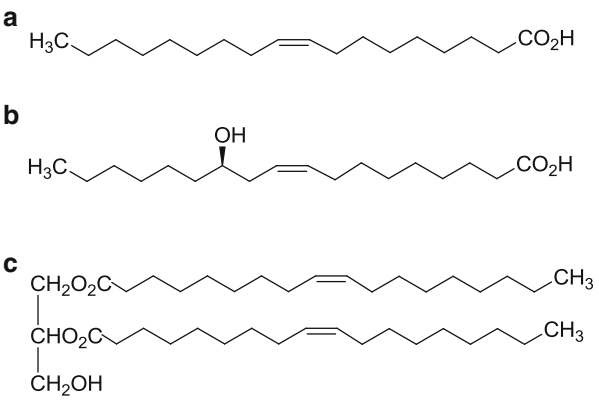

Fig. 3 Chemical structures of oleic acid (**a**), ricinoleic acid (**b**), 1,2-diolein (**c**)

acid (Fig. 3b) in elaiosomes, (Bresinsky 1963) could not be verified by subsequent studies (Marshall et al. 1979; Kusmenoglu et al. 1989; Peternelli et al. 2008).

Regarding their controversial results the findings mentioned above have to be discussed in some detail (Marshall et al. 1979 versus Bresinsky 1963). In subsequent investigations since 1963 it could be demonstrated that diglycerides with unsaturated oleic acid as component (1,2-diolein; Fig. 3c) are important in relation to myrmecochory. According to these investigations diolein is widely distributed in elaiosomes and in higher quantities than other types of lipids. It stimulates certain species of ants (*Aphaenogaster* spec.) to pick up and transport diaspores with elaiosomes (Marshall et al. 1979). The results have been confirmed in some subsequent studies. On the other side it became apparent that in some of the myrmecochorous plants in North America (*Sanguinaria canadensis*, *Trillium sessile*, *Dicentra cucullaria*) the quantities of diglycerides were inconspicuous as compared to the content of triglycerides. Out of four investigated species only in one of them (*Jeffersonia diphylla*) the quantity was high enough for detection of 1,2-diolein in their elaiosomes (Kusmenoglu et al. 1989). In some subsequent studies it was reported that the elicitors for uptake and transport of diaspores through ants are obviously not the diglycerides but rather free fatty acids, especially oleic acid (Boulay et al. 2006; Pfeiffer et al. 2010). Further, one has to consider that triglycerides and diglycerides presumably split off fatty acids; this might happen regularly in the presence of lipases.

In my own investigations a simple method which was available at that time (1960) had been used for the separation of fatty acids following Nowotny et al. (1958) (chromatography on petroleum impregnated paper with the solvent glacial acetic:water 8:1). Applying this method the saturated fatty acids remained at or near the starting point. Also the triply unsaturated linolenic acid was not migrating at a considerable longer distance, whereas ricinoleic acid followed close to the front line and turned black if treated with vapours of osmic acid (OsO_4: an unspecific reaction with the double bonds in alkyl-chains). Using such a rather crude procedure of separation also other hydroxylipids would show similar behaviour as ricinoleic acid. Therefore this kind of proof was taken as ambiguous and

preliminary (Bresinsky 1963; see p. 27 and Fig. 79) in regard to the elaiosomes of *Melampyrum*, *Melica*, *Luzula*, *Veronica* and *Viola* as well as for the larvae of a species of ants.

In order to verify the then assumed occurrence of ricinoleic acid in elaiosomes gas chromatography was applied taking the elaiosomes of *Melica nutans* for investigation. The experiment was performed by A. Prox at the Institute of Organic Chemistry at the Technical University in Munich. Lipids were extracted from the elaiosomes of *Melica nutans* and the free fatty acids were methylated. The run of the extracted and methylated fraction of lipids demonstrated the occurrence of free ricinoleic acid without doubt. Additionally the ant's response to ricinoleic acid was tested in a laboratory nest with the species *Lasius fuliginosus*. Small squares of filter paper soaked with a solution of ricinoleic acid (0.01 %) and also test objects with some other fatty acids (incorporated in the same manner on squares of filter paper) were exposed in an area outside the centre of the nest. Paper squares with ricinoleic acid found vivid interest by the ants and were picked up and transported at a higher rate than paper squares soaked with oleic acid or linolenic acid or the control (bar squares of filter paper; Fig. 4). However, squares cut from the chromatogram in the area of ricinoleic acid were ignored, presumably because it was not possible to remove all of the solvent and of the petroleum impregnation from the chromatogram paper.

Ricinoleic acid is to be distinguished from oleic acid by the presence of a hydroxygroup at C-12 (compare Fig. 3a, b); otherwise the structure of both fatty acids, each with a double bond at C-9, is identical. Even in the investigation of Marshall et al. (1979) it became evident that most of the antennation reactions of *Aphaenogaster* spec. came about in relation to test objects with ricinoleic acid; however, transport of test objects was observed only if they were treated with 1,2-diolein. Several saturated fatty acids (with a shorter carbon chain than ricinoleic or oleic acid) serve the function of trail pheromones in *Lasius fuliginosus*, indicating that chemoreception in this species is largely based on a variety of different fatty acids (Huwyler et al. 1975). Nine saturated or non-saturated fatty acids with a longer chain (e.g. C_{14}, C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$, $C_{20:4}$) than in the case mentioned above are the components of the trail pheromone of the ant species *Pristomyrmex pungens* (Hayashi and Komae 1977); the identified $C_{18:1}$ fatty acid is similar to or identical with oleic acid.

Without restricting the validity of the results of Marshall et al. (1979), it has to be stated that comparability of results depends on using the same species (of ants and plants) under standardised conditions of the test procedure. Moreover, subsequent studies of several investigators have shown that it were not in all cases diglycerides with oleic acid as component, even not always free oleic acid, which released transport activity of the ants (Lanza et al. 1992; Gammans et al. 2006). Further it was shown that the lipid composition in elaiosomes may depend on the time of seed ripening and on the degree of maturity (Peternelli et al. 2008).

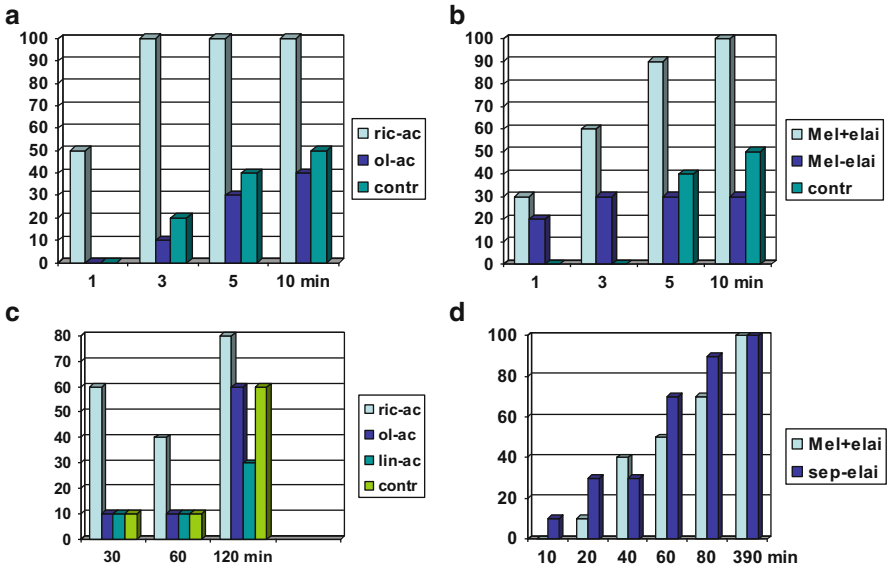

Fig. 4 Transport of diaspores and of squares of filter paper incubated with fatty acids. Ordinate: share of transported items out of 10 exposed items; 60 means 6 of 10 items transported. Abscissa: time of observation in minutes. (**a**) Test with squares of filter paper incubated with ricinoleic acid (ric-ac) or oleic acid (ol-ac) as compared to the control (squares not incubated) in a laboratory nest of *Lasius fuliginosus* (Bresinsky 1963). (**b**) Transport of diaspores of *Melica nutans* with elaiosome (= Mel + elai) and without elaiosome (= Mel − elai) as compared to blank squares of filter paper (= contr) in a laboratory nest of *Lasius fuliginosus* (Bresinsky 1963). (**c**) Test with squares of filter paper incubated with ricinoleic acid (ric-ac) or oleic acid (ol-ac) or linolenic acid (lin-ac) as compared to blank squares (not incubated = contr) in a laboratory nest of *Lasius fuliginosus* (Bresinsky 1963). (**d**) Field experiment with diaspores of *Melica uniflora*, exposition of diaspores with elaiosome (= Mel + elai), separated elaiosomes (= sep-elai). Not any of the control items (diaspores without elaiosomes) were transported by ants during the whole time of the experiment (Sernander 1906)

2.4.2 Sugars

Though being found as regularly occurring compounds in the elaiosomes of nearly all of the investigated species in significant concentrations, sugars in elaiosomes (Bresinsky 1963) did not find much attention in later studies. Neglect of this aspect up to the present (except in Fischer et al. 2008) is hard to understand. First vague assumptions in respect to the presence of sugars in elaiosomes have been given by two of the early authors (Ulbrich 1919; Uphof 1942). Sugars in elaiosomes are in the order of their frequency glucose, fructose, sucrose and rather rarely xylose (Bresinsky 1963). Ants are not interested in the occurring pentose xylose, whereas the concentrations of hexoses (glucose, fructose) and those of the disaccharide (sucrose) in elaiosomes are high enough to draw their interest on. The concentration of the sugars in elaiosomes is much higher than their threshold value. In some of the elaiosomes the concentration of sugars is even high enough (*Iris ruthenica*, *Galanthus*) to be noticed as sweet taste by the less sensitive human tongue.

A special case is realised in *Primula vulgaris*. The seeds of this plant are equipped with elaiosomes. The diaspores are exposed in capsules opening close above the ground. The elaiosomes contain lipids, and glucose in low concentration just to be detectable. The seeds get separated from the central placenta at the time of fruit maturation. They remain in dense clusters within the opened capsule. At this time the placenta secretes a liquid containing sucrose, glucose and fructose. Again the concentration of the diluted sugars is high enough to be noticed by the human tongue. Finally the seeds get coated by the sweet liquid like a frosting. These observations agree with the fact that the just opened capsules of the plant (still fixed to it) are already visited by ants (Sernander 1906; Bresinsky 1963) and that even seeds with removed elaiosomes are transported by them (Sernander 1906). Non-myrmecochorous species of the same genus *Primula* (*P. elatior, P. veris*) with fruits on elevated stems have no elaiosomes and the placenta does not secrete a sweet liquid containing sugars.

The role of sugars in elaiosomes in comparison to lipids has to be tested experimentally. Appropriate experiments remain to be done. Judging from the occurrence of lipids and sugars in elaiosomes in regard to their respective attractiveness to ants, only indirect conclusions can be drawn based on the following observations. As elaiosomes of some myrmecochorous plants contain lipids, but no sugar (as in *Chelidonium* spec.), and on the other side some non-myrmecochorous species have seeds with appendages that contain sugar but no lipids (*Colchicum* spec.) it can be deduced that sugars alone, if not present in higher concentrations (i.e. not above the threshold value to a considerable extent), do not contribute essentially to myrmecochory. However, if the concentration of sugars in elaiosomes and on the seed coat is high enough, then myrmecochory might be triggered by the presence of sugars alone as in *Primula vulgaris*. In the elaiosomes of *Melica nutans* no lipids in form of triglycerides were found histochemically, but sugars were detected. Nevertheless, the situation in *Melica nutans* should not be compared with the situation in *Primula vulgaris* because lipids are obviously present as free fatty acids in elaiosomes of *Melica* (Bresinsky 1963).

2.5 Advanced Studies on Myrmecochory

The studies which followed the publication of Marshall et al. (1979) brought to light several new aspects in regard to myrmecochory.

Ants react differently if they are confronted with diaspores of different types with or without elaiosomes. Their behavioural pattern includes ignoring, eating the elaiosome in situ, transport of the diaspores directly to the refusal area of the ant's nest, transport into the nest (foraging, feeding), or information of other workers for support in transport activity (recruitment). For each of these behavioural reactions special releaser compounds are postulated. The fate of a diaspore after coming into contact with an ant would more or less strictly depend on specific chemical elicitors in the elaiosome which cause a special reaction such as eating, feeding, necrophoric

behaviour, foraging or recruitment (Bradshaw and Howse 1984). This idea is contradicted by the observation that two different kinds of transport (necrophoric or foraging) are triggered by oleic acid anyway; its outcome depends on the main current activity in which the ants are engaged in the nest (Gordon 1983).

Diaspores equipped with elaiosomes are carried into the nest by ants for feeding their larvae. After feeding, the diaspores being deprived from their elaiosomes (however, with the intact embryo being still embedded in the seed) are disposed in deeper parts of the nest or on refusal piles outside the nest. In labelling experiments it has been shown that the rate of uptake of nitrogen and carbon originating from elaiosomes is considerably higher in the ant's larvae (61 %) than it is in the workers (39 %) of a colony (Fischer et al. 2005).

The possible advantages for the plant are manifold (Beattie 1985). Different variants of a selective advantage are discussed (Hölldobler and Wilson 1990; Giladi 2006; Fokuhl 2008): directed dispersal of diaspores to suitable places for germination of seeds and for the establishment of progeny, migration over certain distances, protection from losses through feeding animals and some other advantages.

The importance of the diglyceride 1,2-diolein as an elicitor of transport activity was also confirmed in case of the ant species *Pogonomyrmex rugosus*. This observation agrees well with the fact that the mentioned compound is present in the elaiosomes of the North American *Hepatica americana* (Skidmore and Heithaus 1988). Lipid fractions obtained from the elaiosomes of this plant were tested in a bioassay using the mentioned ant species. The distances of diaspore transport by the ants were determined. The highest transport rates were observed using intact seeds, isolated elaiosomes, crude extracts of non-polar lipids and diglycerides containing fractions from the TLC separation of lipids. Transportation rates were remarkably lower, however still moderate, if the ants were confronted with test items containing mixtures of polar and non-polar lipids or with free fatty acids (exclusive ricinoleic acid which could not be traced in the elaiosomes of *Hepatica americana*). Transportation rates were definitely low or nearly zero in case of test items containing monoglycerides and triglycerides, or if blank test items (without lipids or other compounds) were offered. The reaction of ants in the time after release of diaspore transport should be differentiated from other behavioural patterns. A possible behavioural attitude of eating immediately would prevent the transportation of diaspores. The release of necrophoric behaviour would only result in removing diaspores out of the nest but not in transport to the nest. Taking this into account there are only two remaining possibilities which could be decisive for the success in dispersal. Compounds within the elaiosomes could release either a behaviour similar to that directed towards the animal prey (arthropods) of ants or a brood-tending behaviour which would also afford a transport activity to the nest (Carroll and Janzen 1973; Horvitz and Beattie 1980; Skidmore and Heithaus 1988). Triolein (triglyceride) and diolein (diglyceride) elicit brood-tending behaviour whereas free oleic acid remains without response in workers of the fire ant *Solenopsis invicta*. Triolein has been identified as a brood pheromone in this ant species (Bigley and Vinson 1975).

The investigation of the differently equipped elaiosomes of three species of *Trillium* (occurring in North America) in respect to their attractiveness towards the ant species *Myrmica punctinervis* permitted an estimation about the value of characters (even if competing with each other) being effective for seed dispersal (Lanza et al. 1992). It is not so much the dimension of an elaiosome, even not a maximal high share of lipids in the spectrum of compounds stored in the elaiosome, rather than the offer of special free fatty acids which is decisive for releasing the transport activity. Oleic acid in higher concentrations present in elaiosomes was shown to promote a strong interest of the ants towards such items. However, higher rates of transport were observed if elaiosomes contained instead of oleic acid a higher share of other free fatty acids. As a matter of fact, linoleic acid was considered to trigger the transportation of seeds (Lanza et al. 1992). It is also linoleic (and linolenic) acid, as a component in triglycerides and as a free fatty acid as well, which is a phagostimulant in the omnivorous ant species *Solenopsis saevissima* (Vinson et al. 1967). These observations and conclusions of the above-mentioned authors eventually limit to some extent the general validity of the results obtained by Marshall et al. (1979).

The response of ants belonging to different nutritional groups towards elaiosome-equipped diaspores is different, depending on the compounds within the elaiosomes. In regard to the fate of diaspores, granivorous ants (like *Tetramorium caespitum*) have to be distinguished from so-called mutualistic species (*Myrmica ruginodis*) which treat diaspores with care and dispersing them without setting damages to the germ. The differences in behaviour of both nutritional groups of ants have been investigated by Gammans et al. (2006) using the seeds of two species of *Ulex*. Different from elaiosomes of most of the other species these are of yellow colour. As expected, the experiments revealed that the colour is not important for the perception of the elaiosomes by the ants. Granivorous ants apparently come into contact only mechanically with the items of their interest. Towards free fatty acids and other lipids no remarkable reactions were observed. Mutualistic seed dispersing ants, however, take interest in the lipids on the surfaces of the diaspores and elaiosomes with the final result of transport. In the *Ulex–M. ruginodis* interrelationship the diglycerides only mediate the first contacts of ants with the diaspores. The elicitation of diaspore transport depends on other compounds with which the ants presumably come into contact after biting into the elaiosomes. Different from the results of Marshall et al. (1979) obtained for *Hepatica americana* the diglycerides of elaiosomes in *Ulex* are not dominated by oleic acid (present only in small quantities) but rather by palmitic acid, stearic acid and arachidic acid. The elaiosomes contain four essential fatty acids (such as linoleic and linolenic acid) and four essential sterols which are required for raising the breed (Gammans et al. 2005).

A variation in the chemical composition of elaiosomes is not only apparent in different genera or species of myrmecochorous plants but also if different provenances within a species are compared to each other (Boulay et al. 2006; Boieiro et al. 2012). In *Helleborus foetidus* the quantities of triglycerides, diglycerides and free fatty acids were different in elaiosomes from distant

(750 km) growing populations (Boulay et al. 2006). The variation was correlated with different attractiveness of the diaspores in respect to ants. The elaiosomes originating from one place were more attractive to ants, especially towards the ant species (*Formica lugubris*), showing a high activity in that area, as compared to the elaiosomes of the other locality with other species of ants (*Aphaenogaster iberica*, *Campanotus cruentatus*) being there most frequently engaged in transport of diaspores. The elaiosomes of higher attractiveness were characterised by their somewhat bigger dimensions (so also according to Mark and Olesen 1996) and by a higher share of oleic acid as compared to the share of palmitic acid and linoleic acid, whereas the share of diglycerides remained equal. In a bioassay dummies soaked with triglycerides, diglycerides or free fatty acids were evidently preferred to be transported by *Aphaenogaster iberica* as compared to the control (only treated with the solvent). Dummies treated with extracts of triglycerides or with diglycerides were not differently transported by the ant species.

On the other side in a pairwise competition test, dummies treated with free fatty acids obtained from the attractive elaiosomes were transported at a higher rate than those with fatty acids from the less attractive ones. The higher attractiveness is obviously associated with the fraction of free fatty acids and is presumably due to a higher content of oleic acid (in the fraction of free fatty acids and also as component of triglycerides and diglycerides). These observations point to regionally differing selective pressures in regard to the chemical composition of elaiosomes (Boulay et al. 2006). Putative adaptations of the dimensions and of the weight of diaspores of *Helleborus* in regard to the size of the involved species of ants were not clearly evident (Garrido et al. 2002).

An indirect evidence for the adaptive value of elaiosomes in regard to the dispersal of diaspores has to be seen in the fact that the composition of compounds accumulated in elaiosomes on one side and in the seeds (without elaiosomes) on the other side is quite different (Boulay et al. 2006; Fischer et al. 2008; Boieiro et al. 2012). The share of myrmeco-attractive lipids in elaiosomes (e.g. oleic acid) and the concentration of soluble carbohydrates (fructose, glucose, sucrose) are higher in elaiosomes than in the corresponding seeds. Differences in composition were also observed with regard to the contents of free amino acids, especially regarding the share of histidine with a 7.5 times higher concentration in elaiosomes as compared to the seed (Fischer et al. 2008). Taking these results into account the nutritive value of elaiosomes as source of nitrogen might be much higher than previously accepted (Bresinsky 1963). The significance of elaiosomes for nursing the brood of ants (as stated by Beattie 1985 for ant colonies in general) was recently proved in experiments (Gammans et al. 2005; Fokuhl 2008; Fokuhl et al. 2007, 2012). A mutualistic interrelationship between ants and myrmecochorous plants is also somewhat supported by the observation of a higher germination rate of seeds (f. i. in 8 of 28 tested species) after having been deprived of their elaiosomes (Horvitz and Beattie 1980; Fokuhl 2008).

An excellent survey on myrmecochory (Mayer et al. 2005) summarises the still existing demands on future research as follows: (1) What is the effect of elaiosomes on ant-colony demography? (2) What is the effect of ant dispersal on plant species

composition? (3) What is the effect of habitat disturbance including invasive species on ant and plant species composition? From my own point of view on the subject the question has to be added: (4) What are the chemical cues (single compounds or several interacting ones in regard to different species and nutritional groups of ants) which are decisive for the uptake and transportation of diaspores under various circumstances?

2.6 *Mimicry and Dispersal in* Melampyrum

At the end of this chapter the mimicry theory with regard to elaiosomes has to be commented briefly. Some reflexions in this context have been made because of some resemblance of the diaspores of *Melampyrum* (Fig. 1) with the pupae of ants (Lundström 1887). Such a similarity in shape remains still surprising enough if the larvae of the ants are considered before transformation into the stage of a pupa. In a realistic view on this phenomenon the existence of some kind of morphological mimicry should be excluded, because the orientation of ants in their environment is not based on visual perception. However, the existence of chemical stimulants being present in the brood of ants and in diaspores as well, releasing an ant-specific behaviour in both cases, could be interpreted as chemical mimicry. Guided by such considerations it was obvious to look for chemical matches (or similarities) between the excretion or surface compounds of the ants' larvae and the elaiosomes of *Melampyrum*. The results obtained at that time (Bresinsky 1963) were that the same lipid (unsaturated fatty acid; eventually mistaken as ricinoleic acid) in larvae and in elaiosomes is eliciting brood-care and seed-transport behaviour. From today's point of view the identification of the substance as ricinoleic acid is uncertain and has to be re-investigated (Marshall et al. 1979 versus Bresinsky 1963). Anyway, proof has been given to the fact that fatty acids are triggering some kind of a specific behaviour of ants which results in seed dispersal and perhaps also in brood-care.

In his experiments Sernander (1906) could demonstrate that diaspores of *Melampyrum* are carried away by ants with a somewhat weaker result than in case of *Polygala*, *Anchusa* or *Centaurea*. It were not only the diaspores with adherent elaiosomes being transported but also seeds with a removed integument cap and seeds after separation of their elaiosome. Of course, also the isolated elaiosomes were taken up by the ants (Sernander 1906). Taking into account that lipid-containing cells are not restricted to the elaiosome and to the integument cap only (see Fig. 2e), this seems to be quite understandable. All these observations demonstrate that the myrmeco-attractive appendages may act not only as means for dispersal but also as protective devices of diaspores against ants.

In more recent studies it was demonstrated that dispersal of *Melampyrum* seeds is decisively supported through the action of ants (Gibson 1993; Heinken 2004). In the dissemination procedure without external influence (barochory) only distances up to 25 cm can be bridged. Actually, however, the seeds are dispersed in a distance

of more than 90 cm on the average and of over 6 m per year at maximum. In this way species of *Melampyrum* are established as locally expanding populations through occasional long-distance transport of diaspores and subsequent regularly performed myrmecochorous short-distance dispersal, even at localities where the plant was missing before. Gaps in the distribution pattern remain often persisting because of less effective long-distance dispersal. In subsequent observations over a time of several years in the course of seeding experiments it could be shown that newly established plants grew predominantly near the nests of ants (Heinken 2004). The observation of the seed transport by ants resulted in finding some new nests of ants which had not been seen before. Seeds of *Melampyrum lineare* being disposed by ants on the ground revealed a higher rate of germination, better viability of the seedlings and a higher fertility of the progeny as compared to seeds artificially sawn (investigation performed in North America by Gibson 1993). Due to hemi-parasitism in *Melampyrum* the disposal of seeds on suitable locations by ants is important. The ramifying germs have to get connected to the roots of a compatible host plant in order to establish sufficient water supply necessary for survival of the seedlings (Gibson 1993).

In the context of mimicry some other results are of interest. Oleic acid stimulates workers to remove the corpses of dead ants and the disposal on refusal piles inside the nest (Wilson et al. 1958). Thus a mimetic release of necrophoric behaviour through oleic acid in elaiosomes may be suggested. Moreover, the possibility has to be considered that elaiosomes mimic prey items, thus ensuring the removal of seeds to the nests of carnivorous ants (Carroll and Janzen 1973; Beattie 1985; Hughes et al. 1994). The fatty acid composition of elaiosomes was found to be similar in the insect prey of ants, therefore attracting omnivorous and carnivorous species as well (Hughes et al. 1994).

In the Neotropics epiphytic clusters of plants (ant gardens) are principally formed by directed dispersal of seeds to ant nests where larvae feed on seed attachments (like arils), or on the pulp or on a sticky gelatinous matrix of diaspores (Davidson 1988). Special volatile compounds on seeds of such epiphytic plants presumably trigger as non-nutritional cues (by stimulation of brood-care behaviour?) dispersal and deposition of the diaspores on suitable places in ant gardens. Although a number of substances present on epiphyte seeds had been tested, no clear indication was found to support such an assumption. The only compound (2-hydroxy-3-methoxy-benzenemethanol) stimulating transport of test items was not present on epiphyte seeds (Davidson et al. 1990).

In a recently published study (Pfeiffer et al. 2010) chemical mimicry is discussed in some other context. The term mimicry is applied on convergent development within one major group of organisms (here independent development of structures of sometimes unequal effectiveness to ensure myrmecochoric dispersal). In the context of possible mimicry of *Melampyrum* seeds one is inclined to think rather of a mimetic disposal of compounds in the elaiosome in order to release a certain social behaviour (brood-care, necrophoric behaviour) favouring the dispersal of seeds. However, there is no valid proof available for accepting chemical mimicry in case of *Melampyrum*, not to mention morphological mimicry (Sernander 1906; Morton 1912).

3 Patterns of Plant Distribution

The adaptive value of the various dispersal mechanisms is apparent. The benefit for the plant is that the progeny is prevented from growing up in a competitive situation with the parental generation and to increase the chance to establish new populations at remote places previously not being occupied. Among many other factors the dispersal mechanisms are decisive in regard to the resulting distributional areas occupied by the plant species in a regional or global scale.

3.1 Dispersal and Distribution

Dispersal is a prerequisite of distribution. The interpretation of distribution patterns is difficult to accomplish because a variety of different factors contribute to the formation of a specific area of distribution within a more or less long (geological) period. However, in a small scale of space and time the effect of dispersal of myrmecochores is well demonstrated in situ. Sernander (1901, 1906) contributed first observations in this regard. In the neighbourhood of ant nests myrmecochorous plant species (e.g. *Luzula pilosa, Melampyrum pratense, Danthonia decumbens*) are often found to grow in lines marking (sometimes abandoned) routes of the ants. These lines are quite regularly leading to places of left ant nests. The ants (e.g. *Lasius fuliginosus*) change their streets quite often, even in summer, and this is one reason for the spread of myrmecochores also in a square dimension around the neighbourhood of their nests (Bäumler 1984). Myrmecochores are often growing in wall joints or as epiphytes (*Chelidonium majus, Hepatica nobilis, Lamium album, Luzula pilosa*) on trees on which they are transported by ants.

3.2 Areal Expansion of Myrmecochores

The distribution patterns of plants do mostly not convey a clear impression about the influence of their dispersal devices on their present-day shapes of areals. It remains difficult to draw conclusions, even in the area of Central Europe where many plant species had to re-migrate during a time of only 10,000 years in the recent postglacial period.

Quite an instructive example is given in case of different species belonging to the genus *Melica* (a genus of grasses; Fig. 5), some of them with myrmecochory (*M. uniflora, M. nutans, M. picta*) enabling short-range dispersal, other ones with anemochory (*M. ciliata*) suitable for long-range dispersal. The distribution maps of these species for Bavaria (Schönfelder and Bresinsky 1990; continued currently in BIB) demonstrate at a first glimpse that the myrmecochorous representatives occupy a larger area (except. *M. picta*) than the anemochorous species does

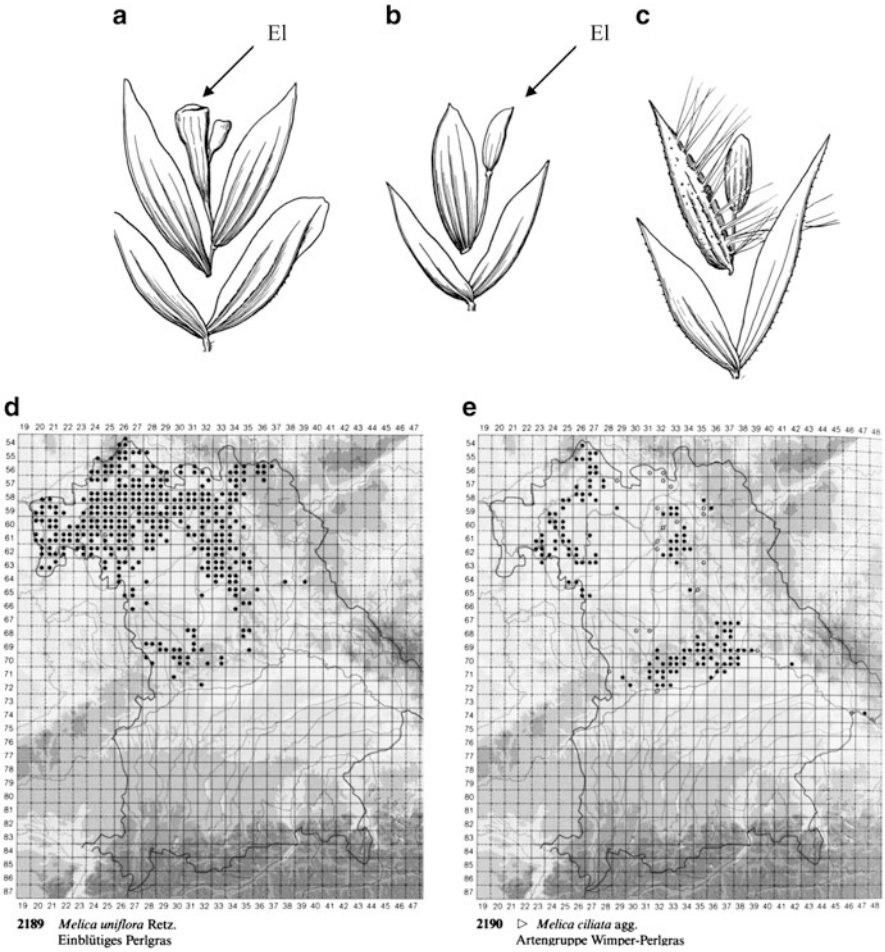

Fig. 5 Myrmecochorous diaspores as compared to anemochorous diaspores of different species in *Melica*. Myrmecochorous: (**a**) *Melica nutans*, (**b**) *Melica uniflora*. El = Elaiosome. Anemochorous: (**c**) *Melica ciliata* s. l. with lemma covered at the margin with hairs as flying devices. (Conert 1998) (**d**) Distribution of myrmecochorous *Melica uniflora* and (**e**) anemochorous *Melica ciliata* s. l. in Bavaria. *Open circles*: records before 1945/1946 (Schönfelder and Bresinsky 1990)

(Fig. 5). An explanation may be seen in special habitat requirements differing largely between the anemochorous species versus the myrmecochorous species. The anemochorous *M. ciliata* requires sunny places on (mostly calcareous) rocks in order to get new populations to be established. Although having quite effective means for long-distance dispersal by wind the species was not successful in colonising all of the isolated potentially suitable rocky habitats. Starting from remote refugial areas during the glacial period (for European distribution see map in Meusel et al. 1965) the settlement on many (but not of all the) isolated bedrock

habitats became possible due to the effective means for long-distance dispersal; depending on myrmecochory instead of anemochory this would not have been managed in this way.

The distributional range of *M. uniflora*, a myrmecochorous species growing in beech forests, shows a conspicuous limit towards the Southern part of Bavaria. Apart from hardly definable climatic factors the explanation for this phenomenon might be seen in unsuitable poorer soils dominating South of the Danube River (in areas with an geological underground originating from the Tertiary period) and in the fact that slow-speed myrmecochory prevented the species from migration into the foothills area north of the Alps in Southern Bavaria (Bresinsky and Schönfelder 1977). A corresponding limit of range, depending on similar reasons, is to be found in Northern Germany, in the area of the former ice cover of the glacial period. It is remarkable that *M. nutans* shows a stronger limitation in distribution there than *M. uniflora* does (Haeupler and Schönfelder 1988; Benkert et al. 1996). A comparison of the means for dispersal in the three myrmecochorous species of the Central European flora reveals that *M. nutans* is equipped with the biggest elaiosome and *M. picta* with the smallest one; compared to these species *M. uniflora* possesses a medium-sized elaiosome (Conert 1998). In correlation with these obvious differences the recolonisation of formerly glaciated (or at least climatically influenced) areas took place with different speed and success: the limits of range are more evidently marked in *M. picta* and in *M. uniflora* as compared to those of *M. nutans*.

Another example is given by *Hepatica nobilis*. In order to explain an obvious limitation of range in the area of the Swabian-Franconian Alb ("Schwäbisch-Fränkische Alb") an uncompleted postglacial recolonisation had been postulated (Gauckler 1939 cited by Meusel 1943). Due to its myrmecochory this plant is a slow-speed migrant which could indeed explain the gap in its distribution. However, for good reasons one should not prematurely rely on a simple deductive-historical interpretation, as long as no thorough evaluation of the environmental factors has been carried out (Meusel 1943). One of the decisive environmental factors would be a lack of calcareous soils; however, such an argument is to be excluded because calcareous soils are occurring widespread in the area of the distribution gap and other plants with similar ecological requirements are not limited in the same manner especially if they are equipped with long-distance dispersal mechanisms (*Anemone sylvestris*, *Pulsatilla vulgaris*; Schönfelder and Bresinsky 1990).

A calculatory approach to the effectiveness of long-distance dispersal (like anemochory) in comparison to short-distance dispersal (like myrmecochory) gives some further insight. In the frondose woods (definition in Sect. 2.2) of Bavaria 70 % of species with a nearly gapless overall distribution are equipped with long-distance dispersal mechanisms and, on the other side, 50 % of the species with remarkable distributional gaps rely on short-distance dispersal only (Bäumler 1984).

3.3 Interpretation of Plant Distribution: Options and Limitations

The interpretation of distributional areas of plants depends on the evaluation of various interrelationships and by that way on criteria of plausibility which one is not be able to prove experimentally in general. Besides the primarily important environmental factors and the ecological demands of plants the time factor plays a certain role, even if a priori quite elusive. The time factor is essential if still migrating plant species do not yet occupy their potential area of occurrence (uncompleted areal formation) or because species are not able anymore to invade suitable ecological niches in a certain area because they are already occupied by competitive species. In the past, patterns of distribution were often attributed to special routes of migration and to strategies of dispersal, usually in a speculative manner of reasoning. This applies to plant species with main distribution in the Alps and additional occurrence along the rivers originating from there and flowing through the adjacent foothills and lowlands of Southern Bavaria to join with the Danube River. Such plants commonly had been interpreted as stream-dispersed plants, as so-called "Schwemmlinge". However, the ruling causes responsible for distribution are much more complex and cannot be explained sufficiently and exclusively by hydrochorous dispersal along the rivers (Bresinsky 1965). An expressive example is given by the often investigated species complex of the buckler mustard, *Biscutella laevigata* s. l. (Fig. 6). Within the polymorphic, not easily to be subdivided species complex the tetraploid subspecies (ssp. *laevigata*; $4x/2n = 36$; x = chromosome base number; $2n$ = diploid phase in life cycle) exhibits a sub-recent tendency to colonise the youngest alluvial gravel terraces of the Lech river whereas the diploid subspecies (ssp. *kerneri*; $2x/2n = 18$) grows on the older gravel terraces along the same river. In the first case the plant (ssp. *laevigata*) migrates from the Alps by means of transport through streaming waters to the lower areas. In the second case the plant (ssp. *kerneri*) came into the region from the residual areas in the Swabian-Franconian Alb (outside the glaciation) along the same river, but migrating stream-upwards in the postglacial period (Bresinsky and Grau 1970). Thus, the river Lech with its calcareous gravel deposits functions as kind of an either-way bridge between the northern range of the Alps and the Swabian-Franconian Alb, linking limestone areas in the South with those in the North and vice versa. The means of dispersal hereby used are only partly hydrochorous.

Herbals characterised by resistance against low temperatures and short vegetation periods could overcome the glacial period in parts of Central Europe in between the glaciated areas in the North and in the South. *Sesleria albicans* with a present-day distribution in the Alps at elevations up to 2,500 m above sea level is an example for surviving in the neighbourhood of glaciers. However, this must not to be confused with the concept of glacial relicts. The latter applies to species which were not threatened rather than promoted during the glacial period and survived under the prevailing present-time conditions of a milder climate in merely isolated populations (Bresinsky 1965, 1993; compare, however Reisch et al. 2002).

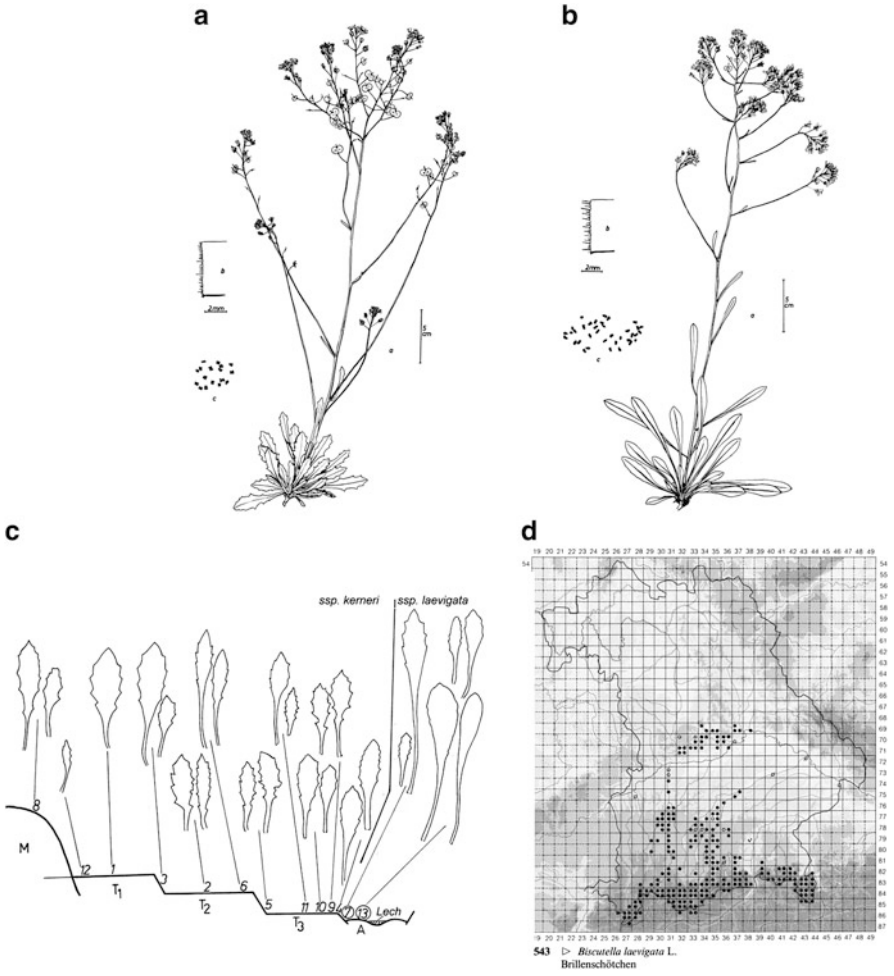

Fig. 6 (**a**) Diploid *Biscutella laevigata* ssp. *kerneri* (prealpine) and (**b**) tetraploid *Biscutella laevigata* ssp. *laevigata* (dealpine) two closely related subspecies with different occupation of niches on the gravel terraces of the floodplane of the river Lech in Bavaria. At the left of each plant a part of the leaf with its hairy margin and the chromosomes (as seen during mitosis in root tips) are drawn. The bars equal 5 cm (plant) or 2 mm (leaf margin). (**c**) *Biscutella laevigata* ssp. *kerneri* on terraces (T1–T3) and on the adjacent moraine landscape (M); ssp. *laevigata* on the alluvial area close to the river (A). (**d**) Distribution of *Biscutella laevigata* s. l. in Bavaria, including ssp. *kerneri* with main distribution on the Swabian-Franconian Alb (Schwäbisch-Fränkische Alb) and ssp. *laevigata* with main distribution in the Alps. *Open circles*: records before 1945/1946 [(**a**–**c**) Bresinsky and Grau 1970, (**d**) Schönfelder and Bresinsky 1990]

3.4 Documentation of Plant Distribution

The comments given above on the areal formation of plants are based on distribution maps which have been established and published in rather extensive atlases during the last decades. Since 1988 we have a complete survey of vascular plant distribution covering Western Germany for the first time, presented in form of maps (Haeupler and Schönfelder 1988). This publication comprises the area of the nine Federal States of Western Germany. It was initiated under the organisational leadership of Heinz Ellenberg assisted by Andreas Bresinsky, Ulrich Hamann, Ernst Wilhelm Raabe and Foko Weberling and was brought to successful publication by the editors Hennig Haeupler and Peter Schönfelder. The afforded field work was accomplished by 1,000 volunteers (300 of them in Bavaria). A short time later, the distribution atlas of vascular plants of Bavaria followed, based on the same organisational structure (Schönfelder and Bresinsky 1990; continued in an internet presentation as BIB), however, using grid squares as reference units with fourfold finer resolution (so-called MTB quadrants). Finally, an equivalent distribution atlas was published for the area of Eastern Germany (Benkert et al. 1996), again with the cooperation of numerous volunteers working in the field and using, like in Bavaria, MTB quadrants as reference units. An urgent requirement of the present time would be the integration of the data from Western and Eastern Germany to a distribution atlas covering the entire area of Germany. In contrast with the Bryophytes (Meinunger and Schröder 2007) and apart from a small number of fungi (Dörfelt and Bresinsky 2003; Bresinsky and Dörfelt 2008) this aim has not been accomplished for the vascular plants so far, more than 20 years after the reunification of both parts of Germany. Different reasons are responsible, among others, the fact that the mapping of vascular plants in Western Germany was primarily based on MTBs, and not from the beginning on the fourfold smaller reference units (MTB quadrants like in Bavaria or in Eastern Germany). The presentation of detailed maps would require to print the maps in four or five volumes instead of only one which is an expenditure not easily to be managed. These circumstances hamper the idea of presenting all data for both parts of Germany in detail. The challenge in the context of the work done and to be done is to motivate experienced and knowledgeable volunteers for the necessary work in the field, to store electronically the influent data, to compute the data in form of distribution maps and finally to critically prove the results and to revise them if necessary. The aspired success depends on several components, on one side on the motivation of volunteer workers and on the other side on the proper processing of big quantities of influent data. Since millions of data have to be processed, the latter task cannot merely be accomplished on a volunteer basis. In regard to my engagement to promote the mapping of vascular plants in Bavaria it seems to me that I was able to provide structures suitable for both aspects (in my function as a chairman of both Botanical Societies in Bavaria for a period of more than 30 years and as a head of a chair at the University of Regensburg). The significance of recording the distribution of plants in maps has been reinforced by several authors (Ellenberg 1988; Meusel 1996;

Bresinsky 2009a). Apart from a scientific interest in the distribution of plants on the globe and in the ruling factors which are responsible for different patterns of distribution, it is also a public demand to record biodiversity of different regions in regard to inventory and dynamics of species.

4 Character Evaluation and Evolution in Fungi

A change in topic from ants to fungi is not so abrupt as it may appear if it is considered that some ants cultivate fungi in their nests.

4.1 Fungi and Ants

The culturing of fungi by the leaf-cutting ants (Attini) is well known for a long time (Möller 1893). In a broader sense 200 tropical species of ants are belonging to this group and 37 species of them cultivate a fungus in a special way: cutting pieces of leaves, transporting them to the nest and preparing a substrate out of it which is suitable for growing the fungus (Möller 1893; Mueller et al. 1998; Pagnocca et al. 2001). The cultivated fungus or—more likely to say—the included nutrients are the main food source fed to the ant's larvae (Quinlan and Cherett 1979; Bass and Cherrett 1995), as it is also true in the case of elaiosomes. The leaf-cutting ant *Acromyrmex octospinosus* definitely preferred the cultivated fungus to a (albeit small) number of different other species of fungi offered in a laboratory experiment (Quinlan and Cherett 1978). The identity of the cultivated fungus (or fungi) was uncertain for long, depending on the fact that the ants grow the fungus in its imperfect (asexual) stage, and if a new colony has to be founded it is this stage in which the fungus is transferred into a new nest by the queen. The taxonomic identification of imperfect stages (anamorphs), i.e. the correlation with the perfect or sexual stage (teleomorph), was difficult to achieve in former times. Occasionally occurring fruit bodies of an agaric (gilled fungus) near abandoned nests and the comparison of fungal structures of the teleomorph (here the agaric) with that of the anamorph (cultivated fungus) enabled a preliminary generic identification and description of a species named as *Rozites gongylophora* Möller (Cortinariaceae). Later perceived investigations resulted in a revision of this identification (Singer 1986; Hinkle et al. 1994; Doherty et al. 2003). Based on DNA-phylogenetic investigations it became evident that the cultivated fungus is a member of the family Agaricaceae (the family includes also the edible mushroom *Agaricus bisporus*, cultivated by man). Within this family (sometimes also taken in a narrower sense as Lepiotaceae) the ant-cultivated species was assigned to the genus *Leucoagaricus* or by other authors also to *Leucocoprinus* (both genera are closely related to each other). Under the name *Leucoagaricus gongylophorus* (or *Leucocoprinus g.*) an assemblage of several species has to be understood

since DNA-phylogenetic studies revealed some heterogeneity on the species level. However, all of them are members of the same family Agaricaceae even in case of yeast-like appearance (Hinkle et al. 1994). Only one basal group within the leaf-cutting ants (*Apterostigma*) cultivates a fungus belonging to another family which was identified as Tricholomataceae (Chapela et al. 1994).

Actually, the interaction between the leaf-cutting ants and the cultivated fungus is much more complex than it formerly appeared to be. Besides the cultivated species in the mushroom garden also a harmful fungus, belonging to the genus *Escovopsis* (Ascomycetes, Hypocreales), occurs. This species would be a serious threat to the cultivated fungus if the ants would not make use of antibiotics which they obtain from a filamentous bacterium (*Pseudonocardia* spec., Actinomycetes) carried with them. The four different organisms (ant, cultivated fungus, pest fungus, *Pseudonocardia*) developed in a coevolutionary interrelationship. Moreover, also nitrogen-fixing bacteria are kept in the ant gardens in order to warrant a sufficient supply with nitrogen for the cultivated fungus (Beattie and Hughes 2002; Currie et al. 2003; Hölldobler and Wilson 2011).

The available DNA-phylogenetical methods permitted not only the identification of the fungal genus and species being used by the ants in their cultures but also provided a possibility of access to their coevolution. One possibility, one could think of, was that the respective ant species keep their cultivated fungus strongly isolated because of clonal reproduction and transfer of the fungus from one generation to the other. Under such circumstances ant-specific fungal strains should arise as independent units. As a matter of fact, it could be observed that in case of the ant species *Atta cephalotes* the fungi cultivated at distant places (one place was in Trinidad and the other one in Panama) were different in respect to their RAPD profiles indicating genetic differences on the population level (Doherty et al. 2003). The isolation of cultivated fungal strains established between geographically distant regions and between different ant colonies is breaking down from time to time. This occurs in case of a loss of the essentially needed fungus when it is obtained again from outside the own nest, either by getting it in the neighbourhood of an abandoned nest or in a predatory mode from nests of the same or some other ant species (Mueller et al. 1998).

In case of the fungus-culturing termites (which are related to cockroaches rather than to ants) the coevolution led to the differentiation of special genera of gilled fungi which are completely dependent on the termites (*Termitomyces*, Tricholomataceae; Heim 1977).

For the observation of interrelationships between ants and fungi one has not necessarily to go into the tropics. For quite a long time it is known that the ant species *Lasius fuliginosus*, living in hollow trunks of trees, cultivates a fungus called *Cladosporium myrmecophilum* in its carton nests (Lagerheim 1900). It is the ant species with which I made my experiments in order to test myrmeco-attractiveness of elaiosomes and of lipid components (Sect. 2.4). I also tried to find chemical compounds which would explain the interrelationship between the ant and the fungus. However, the attempt was not really successful (except some doubtful indication for the presence of fat and of vitamin C). It could have been that the

expected compounds (lipids, sugars) were not present in the fungal hyphae or, respectively, that the employed methods of extraction were not sufficient enough (Bresinsky 1963). In the older publications (Neger 1913) it had been reported that the hyphal tips of the fungus secrete droplets of water in which the ants are interested. However, it seems to be more likely that the fungus fulfils the function of binding together the ant-prepared material used for the construction of the carton walls of the nest (Lagerheim 1900; Maschwitz and Hölldobler 1970; Hölldobler and Wilson 1990). The material is glued together by a sugary liquid which the ants obtain from harvesting honeydew outside the nest (Maschwitz and Hölldobler 1970). The sugar serves also as a substrate for the cultivated fungus. By grazing, the ants obtain a felt-like lawn of the fungus on the carton with the result that the hyphae interweave more densely the material used for wall construction (Lagerheim 1900). Recently the identity of the fungus was investigated by using DNA-analytical methods. The result was that *Lasius* uses more than only one species of fungi in the carton nests. All of them are members of the Ascomycetes, however, not belonging to the genus *Cladosporium*. A mutualistic interrelationship of *Lasius* was found to be established with two or three fungal species which are related to species of the genus *Anungitopsis* instead of *Cladosporium* (Schlick-Steiner et al. 2008). So far it remains not quite clear whether the fungi occur independently also outside of ant nests. There are some indications that free existing species on wood are at least closely related to the mainly cultured ant fungus (Lagerheim 1900).

The example of the fungus-culturing *Lasius fuliginosus* suggests looking for interrelationships between ants and fungi even in our latitudes, regardless whether these may be of somewhat looser or tighter nature. The occasional fruiting of fungi on or close to nests of ants nourishes such a suggestion. One fungus species was even named as *Entoloma myrmecophilum* alluding to its first record on an ants nest. These occurrences not necessarily are merely due to random. Some of the findings may reflect still unexplored interrelations of whatever nature. It could be that ants participate, occasionally by chance or regularly, in the dispersal of spores or of pieces of mycelia, also in the supply or preparation of substrates not to speak of the use for their own nourishment (Bresinsky 1999). The database PILZOEK was established for retrieval of information regarding such and other ecological interrelationships (Bresinsky and Düring 2001; Bresinsky et al. 2005). According to this database a variety of 33 species of fungi (under the code 35.1 "Ameisenhaufen") have been recorded in close connection with the nests of ants in Central Europe. More often, recorded species belong partly to the same taxonomic order and family (Agaricales, Agaricaceae, *Macrolepiota*) as the fungi of the leaf-cutting ants do; partly they are members of other orders (f. i. genera of the Boletales: *Hygrophoropsis, Paxillus;* Bresinsky 2008; PILZOEK). Also mycorrhizal species are among this group, apparently invading the ant mounds from adjacent mycorrhized tree roots. In summary it can be stated that most of the observed fungi on ant hills are edible or not poisonous for men (except *Paxillus involutus*; Bresinsky and Besl 1985).

4.2 Fungi and Biodiversity

Although unexplored interrelationships between ants and fungi may exist even in temperate regions, such interactions are clearly not as important as other ones are. Fungi and algae or fungi and vascular plants interact with each other in lichens and respectively in mycorrhiza, and these interactions are more common and also more important from an ecological point of view (Fig. 10). Fungi represent a separate kingdom within the eukaryotic organisms as also plants and animals do. Worldwide the number of fungal species is estimated to exceed 100,000. Taking into consideration just the area of Bavaria the number of species in a most diverse group (Basidiomycota) is approximately 4,000, and the total number of fungal species is estimated to be not much less than 10,000 (Besl and Bresinsky 2009; estimation of the total number of fungal species in Bresinsky 2010) compared to approximately 3,000 species of vascular plants (Merxmüller 1965; Scheuerer and Ahlmer 2002). However, it remains debatable if species of fungi represent (at least in the regular case) units which are properly delimited in accordance with the biological species concept.

4.3 Speciation in Fungi

A first step in the evolution of biodiversity is speciation. Mechanisms of speciation are still poorly investigated in higher fungi.

4.3.1 Polyploidy in Fungi

Polyploidy is a common phenomenon observed in speciation of vascular plants as it had been demonstrated for *Biscutella laevigata* here (Fig. 6). Different levels of ploidy are determined by counting the chromosome numbers. In case of fungi chromosome counting is hampered by bad individualisation of the chromosomes as clearly visible units during nuclear division. It means that chromosome numbers are almost not determinable during mitosis and meiosis by use of conventional light microscopy. So one has to rely on an indirect assessment, e.g., by measuring the relative DNA contents in cell nuclei in order to comprehend the amount of genetic material involved (Bresinsky et al. 1987a; Wittmann-Meixner et al. 1989). Comparability of the measured values in different fungal species is only given if the nuclei were measured in the same part of the hyphal system and at a defined stage of the DNA replication during the cell cycle, and if species not being too distantly related to each other are compared. In observing all of these circumstances diploid nuclei ($n = 2x$), tetraploid nuclei ($n = 4x$), decaploid nuclei ($n = 10x$) and so on will have whole multiples of the basic DNA amount (assigned to $n = x$). Instead of relating the relative DNA contents to different chromosome numbers it would be

more feasible to refer to the quantity of genetic material. The terms haploid, diploid, etc. and polyploid are used in the latter sense hereinafter.

Polyploidy in connection with speciation results in higher variability and in the establishment of isolating barriers which prevent (more or less absolutely) hybridisation. The determination of the relative DNA content of nuclei in about 150 different species of the Boletales exhibited polyploidy to be a widely occurring phenomenon (Wittmann-Meixner 1989; Bresinsky and Wittmann-Bresinsky 1995) as it was already known in the case of vascular plants. The same holds true in respect to other groups of fungi, f. i. the Ascomycetes where 500 species were investigated in the same context of research (Weber 1992; Weber and Bresinsky 1992).

Intraspecific heteroploidy (or between more closely related species) indicating an initial process of speciation ("in statu nascendi") was only rarely observed in the investigated fungi. Within the Boletales some species of the genus *Leccinum* may serve as a rare example: *L. scabrum* ($n = 2x$) is to be distinguished from *L. subcinnamomeum* ($n = 3x$) only if some minute features are considered (Bresinsky and Wittmann-Bresinsky 1995). Within the rather high number of investigated species (500) of Ascomycetes only in 7 species heteroploidy was shown to occur (Weber 1992).

In regard to speciation and evolution some conclusions can be drawn from the frequency and distribution of polyploidy. Species on the ploidy level of haploids are regarded to be more basal (less advanced) than species on the diploid or even polyploid level. Basal species are accordingly found to a higher share in refugial areas which had been less disturbed by glaciation in the geological past. A refugial area has undoubtedly to be seen in the Northeast American area of frondose woods. Here the share of haploid species of Boletales was found to be 48 % as compared to glacial-disturbed areas of Central Europe with a share of only 6 % of haploids (Wittmann-Meixner 1989). Mycorrhiza forming species (like *Suillus pungens*) bound to relictic hosts with a restricted area of natural occurrence (like *Pinus radiata*; originally known only from California) are haploid in contrast to tetraploid species (*Suillus sibiricus*, *S. plorans*) which are bound to pines with five needles in a bunch (*Pinus cembra*, etc.) and which are widely distributed in the Northern hemisphere, especially in boreal regions and in subalpine altitudes of mountains like the Alps (Wittmann-Meixner 1989; Bresinsky and Wittmann-Bresinsky 1995). These are examples of speciation whereby large areas of recent glacial disturbances (like the Alps; Northern Eurasia) were preferably invaded by polyploids segregated from diploids and haploids.

Moreover, the phenomenon of polyploidy is connected with evolutionary progressions. Within the investigated Ascomycetes the wood inhabiting (lignicolous) species are represented to a much higher extent on the haploid ploidy level than species affiliated with other substrates. Nutritional specialists on unusual substrates or on definite organs of substrate plants, f. i. on leaves or on fruits of gymnosperms or angiosperms, and species with a short time of fructification are more often represented in higher ploidy levels than species on wood and herbs or than long-time-fruiting species do (Weber 1992).

4.3.2 Breeding Systems Control Speciation

A most important parameter for speciation is given by the ruling breeding system. It was investigated in several species of fungi in respect to speciation. Speciation is promoted by two counteracting procedures in outbreeding species of fungi (with an open breeding system): on one side by promotion of quite unlimited recombination and thereby high output of genetic variability (prior to the evolutionary establishment of a new species) and on the other side through conservation of the achieved genetic character divergence with aid of established genetic isolation mechanisms (genetic isolation). Such a strategy is different from that of inbreeders (with a closed breeding system).

Within the investigated Ascomycetes (33 species of Leotiales, Pezizales, Xylariales) most of the species were characterised by an almost closed breeding system (Weber 1992). Reproduction is performed by autogamy/homothallism, e.g. through pairing and fusion of nuclei within multinucleate cells of the hyphal system. In such cases the genetic variability is restricted to the genetic differences of nuclei being present in higher numbers in the hyphae of a single individual of a fungus species.

Within the investigated Basidiomycetes an open breeding system (heterothallism) was found to be prevailing apart from some exceptions. One of those is to be seen in the genus *Phellinus* s. l. Some species in *Phellinus* are homothallic inbreeders, i.e. operating their reproduction in a closed breeding system, independent of the number of nuclei in the cells, some being dikaryotic (with two nuclei in each cell as in most of the Basidiomycetes) and some of them being oligokaryotic (with several nuclei in each cell; Fischer 1987). Other species within the genus are heterothallic outbreeders. Speciation is associated with specialisation to different host plants or substrates as it is often to be observed in many other species of fungi. The heterothallic species *Phellinus torulosus* f. i. is divided into two intersterility groups without exhibiting differences in their morphology, one of which occurs on the Canarian Islands on Ericaceae and Lauraceae as host plants and the other one on various frondose trees in Southern Europe (Fischer and Bresinsky 1992). *Phellinus torulosus* s. l. appeared to be a suitable fungus for experiments because it is possible to obtain fruit bodies in cultures (as one of the few exemptions in higher fungi). The phylogenetic interrelationships within the Hymenochaetales, to which the genus *Phellinus* belongs, have been exemplified in several DNA-phylogenetic studies which have been performed in our institute (Wagner and Fischer 2001, 2002a, b).

4.3.3 Speciation Within the Genus *Pleurotus*

The genus *Pleurotus* with its approximately 30 species (among them the highly appreciated edible Oyster Mushroom, *P. ostreatus*) represents a group within the Basidiomycetes which is suitable for an experimental approach to phenomena of speciation since their complete life cycle including formation of fruit

bodies can be followed in the laboratory. In respect to the DNA quantity of cell nuclei the investigated species can be assigned to three groups: $1x$ = *P. cystidiosus*; $2x$a (meaning somewhat lower than in $2x$) = *P. calyptratus, P. cornucopioides, P. dryinus*, *P. ostreatus*, *P. pulmonarius*; $2x$ = *P. eryngii*; $3x$ = *P. salmoneostramineus* (Bresinsky et al. 1987b; Wittmann-Meixner 1989). The DNA content of nuclei in the $2x$a level is distributed on 12–14 chromosomes in *P. eryngii*; the chromosome number had been determined by counting the components of the synaptonemal complex using a most laborious electron microscopical technique (Slézec 1984). Accordingly $1x$ would be an equivalent to 6–7 chromosomes, $2x$a to 8–10 chromosomes and $3x$ to 18–21 chromosomes, provided a reliable correlation between the quantity of nuclear DNA and the number of chromosomes. The genomic size of the haploid nucleus expressed by the number of base pairs (1 Mbp = 1×10^6 base pairs) is calculated to be about 16 Mbp on the $1x$ level (*P. cystidiosus*), about 24–27 Mbp on the $2x$a level (*P. ostreatus, P. pulmonarius*), about 35–39 Mbp on the $2x$ level (*P. eryngii*) and about 50 Mbp on the $3x$ level (*P. salmoneostramineus*) (Kullman et al. 2005).

It could be demonstrated that speciation is promoted by a breeding system in *Pleurotus* that restricts intraspecific inbreeding to a considerable extent but promotes unlimited outbreeding due to homogenic incompatibility and multiple allelism of factors controlling the crossing. A scheme explaining this phenomenon is presented in Bresinsky (2008, Fig. 10-58). Genetic recombination between physiologically and morphologically different strains of a species is promoted hereby, followed by a considerable variability (Bresinsky et al. 1987b). The process of speciation is completed if the genetic flow between the strains gets effectively interrupted by isolating barriers (Bresinsky et al. 1987b).

Restriction of gene flow between populations (of different species or of units on the way to become different species) is more or less strict. It might be given by ecological factors like being bound to different hosts, or it might be established in form of genetic barriers. Ecological barriers, physiological divergence and some restriction of sexual intercompatibility are obviously correlated with speciation procedures in the complex of *P. eryngii* (including its closely related satellite species, all of them parasitising on different hosts of Umbelliferae). Contributions to elucidate speciation in the complex of *P. eryngii* s. l. have been performed by a group of researchers in France (Cailleux and Joly 1981) and independently also in our working group in Regensburg (Hilber 1982).

The experimental results obtained for the other species in *Pleurotus* support a narrow species concept practised in the taxonomy of fungi. Even if the morphological divergence between different species is hard to comprehend, as in case of *P. ostreatus* versus *P. pulmonarius,* a complete genetic incompatibility barrier was found to be established separating the species. This holds true for all of the investigated saprotrophic representatives of *Pleurotus* on the species level (Hilber 1982; Bresinsky et al. 1987b) as well as for species in *Phellinus* s. l. (Fischer 1987; Fischer and Bresinsky 1992).

4.4 Diversity of Fungal Metabolites

The tremendous diversity of fungal species can also be seen in a nearly indefinable multiplicity of metabolites (Turner and Aldridge 1983) and in an immense variety of ecological interrelationships and dependences (Beck and Lange 2009; Bresinsky and Ziegler 2009). Fungi seem to be unmatched in their metabolic capabilities. The fact that fruit bodies of fungi accumulate toxic compounds, which are of nearly universal efficiency in some cases as the RNA-polymerase inhibiting toxins in *Amanita* (overview in Bresinsky and Besl 1985), presumably results from the selective advantage in connection with the rejection of pests. A similar function has to be assigned to the bitter compounds in fruit bodies of fungi like the 2*H*-azepines or the calopins in some of the boletes (Sterner et al. 1987; Hellwig et al. 2002). A clear antibiotic function is given in respect to many of the metabolites which are released by fungal mycelia into the substrate. The diversity of fungal metabolites obtained in the course of evolution is definitely bound to the effect of defence against animals (Besl and Blumreisinger 1983; Spiteller 2009), as well as against microorganisms. On the other side some of the accumulated chemical components could be regarded as the result of decomposition of the substrate and the hereby formed breakdown products.

Only a part of the fungal metabolites are pigments which are synthesised in numerous structural variants. For the macromycetes (fruit body forming higher fungi) a comprehensive overview of the great variety of pigments exists (Gill and Steglich 1987) based on to the main metabolic pathways leading to different types of pigments. This work was followed by a number of studies performed in the working group around W. Steglich (e.g. Hellwig et al. 2002; Bröckelmann et al. 2004; Justus et al. 2007), partly also in cooperation with our group in Regensburg (e.g. Besl et al. 1975, 1989). The colour has mostly to be seen as an incidental attribute without functional importance in regard to the antibiotic effectiveness of the compounds.

The evaluation of the occurrence of different types of pigments in fungi demonstrates some specificities in their taxa distribution in genera, families or orders. This also includes, however, the possibility of a distribution in taxa which are not closely related to each other (e.g. anthraquinone pigments in members of the Cortinariaceae and Tricholomataceae; muscaflavine derivates in Amanitaceae and in Hygrophoraceae). It is also possible that a compound expected to be present, as seen from a phylogenetic point of view, may be missing because the pathway leading to the end product is blocked or because a side way of the pathway remains a priori undeveloped. Such circumstances hamper the evaluation of chemical characters for establishing a classification in accordance with phylogeny.

4.5 Pigments and DNA-Sequence Data Support Boletales as a High Rank Unit

The order of the Boletales (it is the order to which the estimated edible bolete *Boletus edulis* belongs, also known under the name cepe) includes among others as a tropical representative the species *Phlebopus tropicus*. Its occurrence and nutrition are reported to be closely affiliated with ants (Gonçalves 1940; Singer 1986). The fungus overgrows colonies of pseudococcids (*Pseudococcus comstocki*; Pseudococcidae are a group within the family of scale insects) establishing a kind of symbiosis with them. The fungus–insect units are dispersed by ants (*Solenopsis saevissima*) to the roots of *Citrus* spec. The fungus and the pseudococcids cause severe damages in cultures of *Citrus* in Brazil. The order Boletales was in the focus of a number of recently published studies using some of the more modern approaches which aim at the establishment of a new taxonomic concept.

4.5.1 Pigments of Boletales

In a first attempt the classical order of the Boletales was chemotaxonomically delimited in our working group using characters such as the occurrence of pulvinic acid derivates, cyclopentanoids and boviquinones (Figs.7 and 9; Table 1). The mentioned compounds will be referred to here, in a somewhat vague manner, as "Boletales pigments". The work would not have been accomplished without the generously granted support of W. Steglich. He has to be regarded as the pioneer in the chemical research of pigments in higher fungi. Some of the Boletales pigments (i.e. some of the pulvinic acid derivatives and the cyclopentanoids) are responsible for the blueing of the flesh of the fruit bodies in the presence of oxidases. The reaction results in the formation of blue-coloured oxides of the mentioned compounds. A scheme of the metabolic interrelationships of the Boletales pigments (Fig. 7) was worked out, partially by using labelled precursors injected into the flesh of the fruit bodies (Gill and Steglich 1987; Steglich W. Metabolic pathways of Boletales-pigments. Personal communication, 2012).

In the context of our own research it became more and more evident that the taxonomic concept of the Boletales had to be considerably widened and to be delimited against other orders (Table 1). This had to be accomplished in an assessment of both morphological features (in spite of their considerable polymorphism) and chemical characters. Fortunately the search for expected compounds was not only done in fruit bodies but also in cultures of mycelia. In this way it became possible to detect or verify relations which make it necessary to include into the Boletales several genera as *Hygrophoropsis* (Bresinsky and Bachmann 1971), *Tapinella* (Gaylord et al. 1970; Gaylord and Brady 1971), *Paxillus* (Bresinsky 1974), *Rhizopogon* (Steglich et al. 1971), *Chamonixia* (Steglich et al. 1977), *Coniophora* (Bresinsky 1974), *Serpula* (Bresinsky 1973), etc. In case of *Hygrophoropsis* the pulvinic acid derivates are present in a methylated form

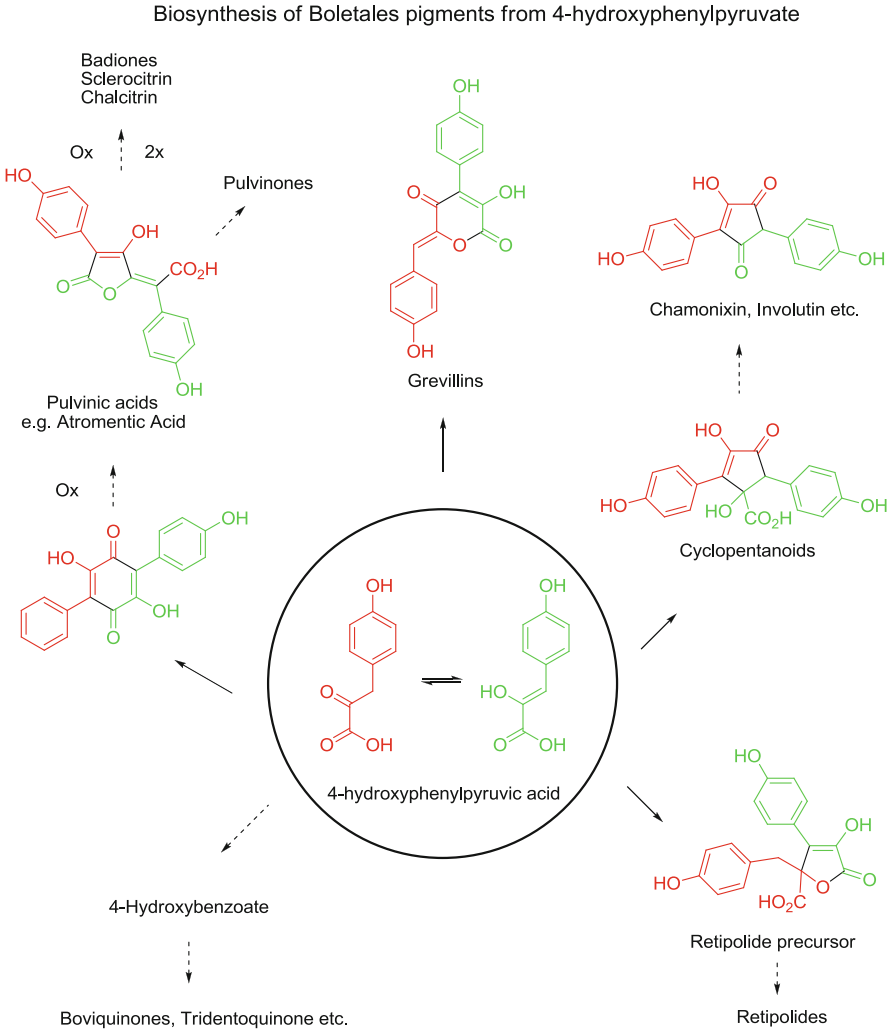

Fig. 7 Survey of the biosynthesis of Boletales pigments (Steglich W. Metabolic pathways of Boletales-pigments. Personal communication, 2012)

(3-*O*-methylvariegatic acid) which may be attributed to the degradation of lignin in the substrate (Besl et al. 1978), although *Hygrophoropsis* as a brown-rot fungus does not decompose lignin as so far known. Meanwhile, methylated compounds were also found in some of the (none lignicolous) mycorrhiza forming boletes such as *Suillus bovinus* (with methylbovinate; Besl et al 2008), suggesting some caution in hypothesising relations between the substrate and the metabolites of fungi.

The presence of a special pattern of compounds (pigments and so on) was occasionally considered as a good additional argument to establish a new genus. In case of the genus *Austropaxillus* from the Southern hemisphere, being formerly

Table 1 Survey of selected taxa in Boletales and Agaricales

Boletales E.J. Gilbert emend. auctores (Agerer, Besl, Binder, Bresinsky, Gill, Hibbet, Jarosch, Steglich)
Tapinellineae Agerer
Coniophorineae Agerer et Ch. Hahn
Austropaxillus Bresinsky et Jarosch
Suillineae Besl et Bresinsky
Suillaceae (Singer) Besl et Bresinsky
Sclerodermatineae Binder et Bresinsky
Boletinellaceae Binder et Bresinsky
Gyroporaceae (Singer) Binder et Bresinsky
Paxillineae Feltgen
Boletineae Rea emend. E.J. Gilbert
Retiboletus Binder et Bresinsky, *Leccinellum* Binder et Bresinsky
Agaricales Clem.
Omphalotaceae Bresinsky
Omphalotus illudens (Schwein.) Bresinsky et Besl
Strophariaceae Singer et A.H. Smith
Leratiomyces Bresinsky et Binder
Hygrophoraceae Lotsy
Porpolomopsis Bresinsky

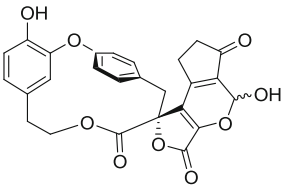

Fig. 8 Structure of retipolide A (Justus et al. 2007)

included in the worldwide distributed genus *Paxillus*, the placement in a genus of its own was actually already obvious because of the strong association with evergreen beech (*Nothofagus*). In comparison to *Paxillus* the pattern of pigments was different too (missing involutin, atromentin, variegatic acid). All these characters including those of the DNA phylogeny (see Sect. 4.5.2) resulted in the establishment of a new genus with a special ecological and geographical background (Bresinsky et al. 1999). Further, the separate position of the genus *Tapinella* against *Paxillus* is supported by the respective pigment patterns (Besl et al. 1989). In case of the genus *Retiboletus* it was the presence of unique compounds named as retipolides (Figs. 7 and 8) which justified the introduction of the new genus *Retiboletus* (Hellwig 1999; Binder and Bresinsky 2002a; Justus et al. 2007). The newly established genus *Leccinellum* is based on anatomical features of the pileus cortex and on pigment characters as well (Binder and Besl 2000; Bresinsky and Besl 2003).

Pigment characters were reasonable criteria enough to establish new families and superorders within the Boletales, e.g. the Suillaceae within the superorder Suillineae with species bound to conifers in establishing a mycorrhiza (Besl and Bresinsky 1997; Table 1). Morphological and anatomical studies with special regard to rhizomorphs revealed some additional characters for the support of new taxonomic positions and subdivisions (Agerer 1999). The main metabolic pathway of pigments in Boletales is directed to the synthesis of their widely spread pulvinic acid derivatives such as atromentic acid, variegatic acid and xerocomic acid (Fig. 7). These compounds are present in "typical" Boletes (Boletineae), in Paxillineae and in Coniophorineae. Besides this favoured branch of pigment metabolism, some other more rarely developed side branches of biosynthesis are special traits in some groups: e.g. within the Boletineae, in *Leccinum* the metabolism to cyclopentanoids and in *Retiboletus* to retipolides; within the Suillineae, the accumulation of grevillins and boviquinones (sometimes in addition to pulvinic acid derivates).

Not more than a few decades ago one had to rely completely on morphological, anatomical and partially also on chemical characters of the secondary metabolism for the delimitation and identification of species, genera, families and orders. The above-discussed examples of approaches in the classification of fungi are largely based on such characters.

4.5.2 DNA Phylogeny

Quite recently DNA-analytical methods became available to elucidate phylogeny on a molecular basis (DNA phylogeny). Previously unimaginable possibilities became available for the identification of not only species but also of populations and strains within species as it had been already exemplified in case of the leaf-cutter ants and the fungi cultivated by them (Sect. 4.1). Moreover, phylogenetic relatedness became a virtually measurable dimension. Different grades of phylogenetic relatedness with all their gradations and interrelationships can now be visually demonstrated in cladograms better than ever before.

These methods, which were quite new at my active time, were used in my working group in order to prove the broader concept of Boletales derived so far from the evaluation of pigments and morphological characters. This was done in regard to the Boletales (Binder 1999; Jarosch 2001; continued by Binder and Bresinsky 2002b; Binder and Hibbet 2006) with the result of a far-reaching congruency in respect to the previously achieved circumference of the order and its subdivision. In few cases only (Omphalotaceae, Scutigeraceae) some unexpected deviations of the results had to be considered. Quite unexpected was the position of the Jack O'Lantern fungus (*Omphalotus*), for which we had established the new family of Omphalotaceae (Kämmerer et al. 1985) within the Boletales based on the capability to synthesise pulvinic acid derivates and cyclopentanoids (Bresinsky and Besl 1979). It is actually not a member of the Boletales judging from results of DNA phylogeny (Jarosch 2001). The fact that the lignicolous,

bioluminescent representatives of *Omphalotus* are different from the rest of the lignicolous Boletales in their capability to decompose lignin (and cellulose; white rot) and to accumulate sesquiterpenes (besides the Boletales pigments) should have been evaluated with more care. The well-supported and distinct family of the Omphalotaceae is not to be included into the Boletales and also not the family of the Scutigeraceae in spite of the accumulation of Boletales pigments in both families (Gill and Steglich 1987). The given situation demonstrates the limitations of a classification based exclusively on chemical characters.

The effort to discover natural relationships and to delimit such units against other groups could be appraised as a merely academic activity without further relevance. On the other side, however, possibilities are provided by such approaches to discover progressions, processes of character differentiation and adaptations which became apparent in the course of evolution. Within the Boletales some progressions are evident: from saprotrophic, lignicolous fungi causing a dry (brown) rot of the *Coniophora*-type to advanced ectomycorrhiza forming symbionts, from association with gymnosperms (conifers) to association with angiosperms, from crustaceous fruit bodies to stalked pileate fruit bodies and finally to closed earthball-like gastroid fruit bodies, from tough, long-living (persistent) fruit bodies to fleshy, short-living (non persistent) fruit bodies and from actively disseminated, wind-borne dispersed spores (anemochory) to passively liberated, animal dispersed spores (zoochory). Regarding metabolism, progressions range from species with a (nearly) complete set of metabolic end products to species with a reduced set, from species with less oxidised metabolites to species with highly oxidised metabolites and so on (Bresinsky and Wittmann-Bresinsky 1995; Agerer 1999). Such progressions linked to a higher degree of diversification are supported by molecular phylogenetic analyses (Jarosch 2001; Binder and Bresinsky 2002b; Binder and Hibbet 2006; see also Fig. 9). A somewhat different picture emerges in respect to the previously stated assumption that in every case gastroid fruit bodies would mark the end stage of a progression (as it remains true for the suborder Boletineae) rather than a basal position. Within the suborder Suillineae a basal position of taxa with gastroid fruit bodies is more likely true than the opposite assumption (Jarosch 2001; Binder and Hibbet 2006).

A comparison of the different natural groups within the Boletales reveals that quite a considerable number of important adaptions were achieved several times independently in the course of evolution (in different phylogenetic lines), f. i. the symbiontic mode of life in form of ectomycorrhiza and the development to the gastroid fruit body (remaining a closed structure within the soil as an adaption to drought and to dispersal of spores by animals). The still highly estimated book "Agaricales in modern taxonomy" (Singer 1986) unites different orders like the Boletales, Russulales and Agaricales within the artificial unit of "Agaricales", and on the other side, includes almost only fleshy fungi with gilled or tubular hymenophores, excluding other morphologies. From the present point of view the book would have to be conceived in a completely different way in regard to the taxonomic delimitation and to the included taxa.

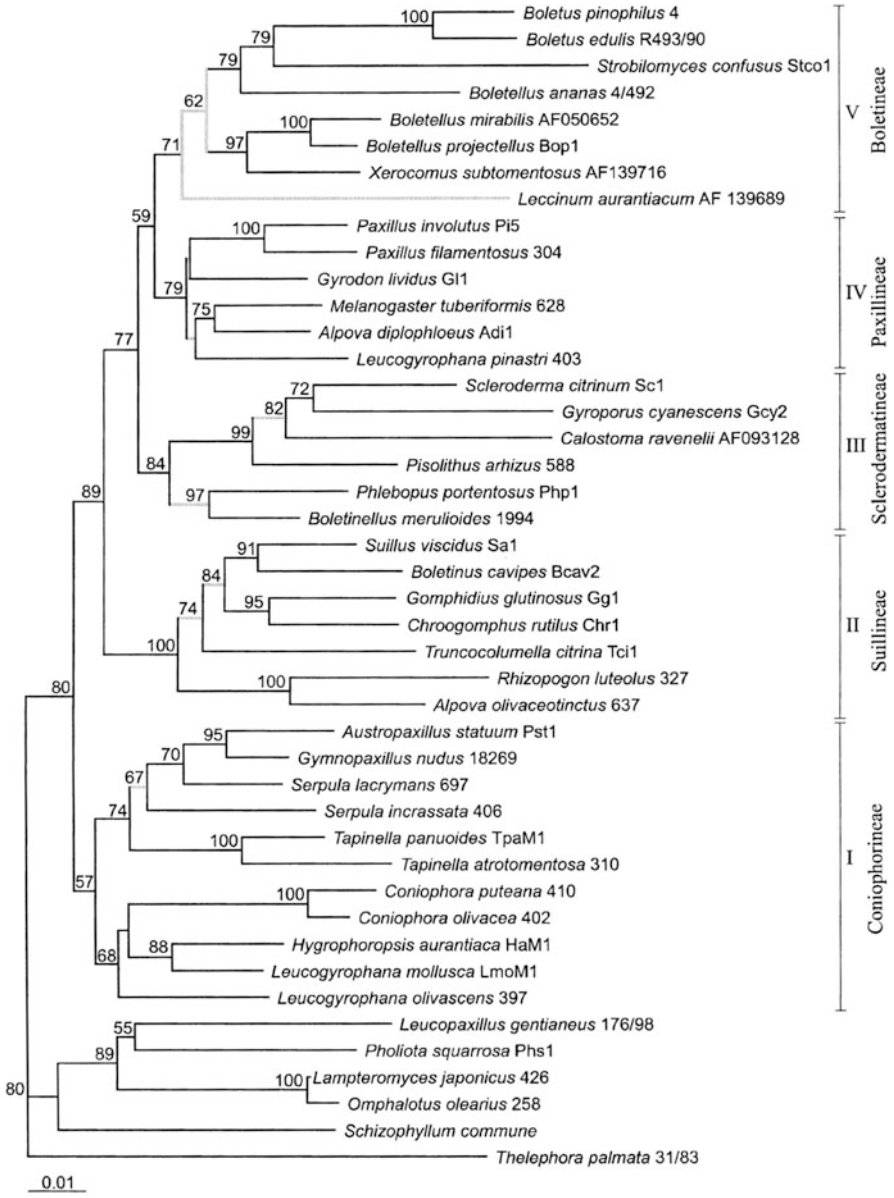

Fig. 9 Cladogram of phylogenetic interrelationships within the Boletales established by DNA sequencing. The suborder Tapinellineae is included in the Coniophorineae here. Phylogenetic evaluation with neighbour joining. Sequences of 900 b from the 5′-end of the 28S gene. Bootstrap values over 50 % are indicated on the respective branches. The bar equals a distance of 1 % (Jarosch 2001)

4.5.3 Concluding Comment

The earlier strategy to ensure phylogenetic coherences of higher ranked groups like orders through the presence of some characters which had to be ideally distributed (individually or in some combination) in all of the included representatives is quite questionable. The limitation of this method is obvious. Today it is the diversification of characters within DNA-explored phylogenies which is in the focus of interest. The question is not so much how interrelationships and phylogenies may be validated or reconstructed by use of characters of the phenotype (taken from morphology, anatomy, cytology and metabolism) but rather how to elucidate various procedures and events of diversification and adaption within the DNA-based phylogenies. In this respect one has to speak of a paradigmatic shift within the scientific branch of systematics.

5 Popularisation of Science

The communication on aims and results of research mainly takes place in scientific journals. Besides such regular efforts it seems to be important to let participate a broader public in scientific approaches. Tax payers, interested people and contributors in some special fields of scientific work demand for information and participation. An outstanding example of a distinguished scientist who also took care of the popularisation of his scientific work was Nobel-laureate K. v. Frisch (1886–1982; Nobel prize in 1973). He was my professor in zoology when I studied in Munich. K. v. Frisch left us not only a body of profound scientific work but also a number of popular books designed for a broader public and a textbook on biology for schools (it was the book I used in school and which was an entry into the secrets of biology for me). From his popular writings his book on animals as builders of constructions should be mentioned here. Among the manifold animal generated buildings which are demonstrated in this book also the carton nest of the ant species *Lasius fuliginosus* (mentioned in Sect. 4.1) is treated (Frisch 1974). Even if the example of K. v. Frisch remains unattainable as a standard, it serves anyway as a kind of justification for a member of a university to address also a broader public according to the limitations and capabilities of one's own personality. Perhaps my commitment to the Botanical Societies in Bavaria can be seen in such a context (Bresinsky 1991b). Hereby the transfer of botanical knowledge to amateurs in an understandable and acceptable manner was aimed for in lectures, field trips and publications. It seemed also to be useful to establish a project of research in which numerous persons outside the university could participate, i.e. the documentation and mapping of plants in Bavaria (Bresinsky 1966). Moreover, it was my intention to report on various topics of our scientific work at the university in an understandable mode such as on the diversity of fungal species in Bavaria (Bresinsky 2010) or about the possibilities offered by DNA-analytical methods to understand the Boletales as a natural group of common phylogenetic origin (Bresinsky 2009b).

6 Protection of Nature

Everybody who gets scientifically interested in biodiversity is called to be committed in the preservation of nature. The goal to protect nature under the economic demands of an industrial society of welfare can be realised in different ways. One possibility is the purchase of areas with a high degree of biodiversity with the aim of its protection. However, the protection of privately owned areas, acquired by organisations which are engaged in nature conservation, might be endangered because of various interventions from outside. As a chairman of the Botanical Society of Regensburg (founded 1790; now the oldest still existing Botanical Society of the world) I had to fight against measurements of the local authorities permitting the nearby pumping off of water (with high content of calcium) which is the basis of the existence for a calcareous marsh ("Sippenauer Moor") privately owned by the Botanical Society (Bresinsky 2005). The result of this fight, even including a dispute pending at the highest Administrative Court of Bavaria, was the installation of support measures against losses of water in the protected area and the establishment of a programme to control the water regime at several water depth gauges and to watch the influences on vegetation composition in a number of squares of permanent observation (Bushart 2006; Scheuerer 2006). There hardly exists another calcareous marsh that would be more intensively investigated in Bavaria than this one. In its sulphurous springs a so far unknown group of Archaea (Crenarchaea) tolerating deeper temperatures was found and a unique association with a *Eubacterium* (*Thiotrix* spec. = Sip 100) was described. The Archaeon possesses a wreath of subtle filaments covered with fine hooks with the aid of which it gets attached to various surfaces. This discovery of a peculiar microbial association which, among others, became possible by previously taken measurements of nature conservation made the "Sippenauer Moor" a hot spot for the research on cold Archaea (Rudolph et al. 2001; Moissl et al. 2005; Koch et al. 2006).

Another aspect of investigation performed in the nature reserve of the "Sippenauer Moor" relates to the estimated number of fungal species (10,000) in relation to vascular plant species (3,000) in Bavaria. As compared to 232 species of vascular plants (Bresinsky 1991a) 680 different species of fungi have been recorded in the area (9.3 ha) within a period of only 1 year followed by an additional year to complete the first achieved results (Krieglsteiner 2002; Simmel 2011). If the time of observation on fungi would have been more extended then the present relation of fungi to vascular plant species (3:1) would certainly have shifted in favour of the fungal species and thus approaching quite well the estimated ratio for the whole area of Bavaria (see also Sect. 4.2).

Based on pollen analysis and on fossil remnants the postglacial history of the vegetation in the "Sippenauer Moor" was investigated in the last doctoral thesis accompanied by me (Petrosino 2006). The initial stages in the development of peat go back as far as to the Late Glacial Period (Allerød period; *c*. 11,000 year BP, i.e. years before present) and it was continued until the present time with differing

growth rates. In spite of several fluctuations (including declines) in peat growth during the Younger Dryas, in the Subboreal and Subatlantic periods the diversity of species was more or less preserved apart from just a few anthropogenic and non-anthropogenic losses. The question whether the biodiversity can be maintained also in future will depend on the effectiveness of the measurements taken to preserve as much as possible of the natural heritage.

7 Teaching

The Latin idiom "*docendo discimus*" (teaching means that also the teacher is learning) is especially valid for the university teacher in regard to his various teaching activities such as laboratory work, lectures and seminars or in escorting the scientific work of students. In this sense I appreciated teaching always as a kind of enrichment. In the context of my teaching activities I participated in shaping the traditional textbook of Botany which had been initiated by E. Strasburger (1844–1912) more than 100 years ago. I was responsible for the treatment of bacteria, algae, fungi, bryophytes and pteridophytes in five editions (ed. 32–36; v. Denffer et al. 1983; Bresinsky et al. 2008). Translations into Italian, Spanish, Serbo-Croatian, Turkish and Russian were subsequently published. An English translation is in preparation. In spite of the international attention which is paid to the book it has now reached a kind of turning point. As seen from its extent and from its claim it is by far not a textbook anymore but rather a voluminous handbook and compendium dealing with the total scope of Botany. Everybody who expects a comprehensive textbook with learnable and didactically presented contents will be disappointed and will criticise its conception (for an alternative approach, see Lüttge et al. 2010). On the other side there is no other book available that would deal with Botany in its complete scope and required fullness including the presentation of prokaryotes and fungi as being traditionally treated within Botany (so in Germany and neighbouring countries). The book should not depart from this completeness because this standard represents its unique profile. Even if it has to be accepted that fungi are a group of organisms of their own apart from the plants, the discussed elimination of most of the text applied to the fungi seems to be problematic because such an approach includes the risk of complete elimination of fungi from academic teaching, at least in Germany where mycology is not an academic subject of its own. An advantage of the book in its present shape eventually is that it deals with organisms which are involved in manifold interactions decisively contributing to the structure and survivability of plants. Such interactions have to be discussed in the context of phylogeny, physiology and ecology of plants (Fig. 10; see also Fig. 10-8 in Bresinsky et al. 2008 based on Bresinsky and Kadereit 2006). The endosymbiontic theory suggesting one of the most important interactions between completely different groups of organisms was initiated by A.F.W. Schimper (1856–1901). He also studied interactions between plants and ants in the tropics (Schimper 1883, 1888) and he was one of the founders

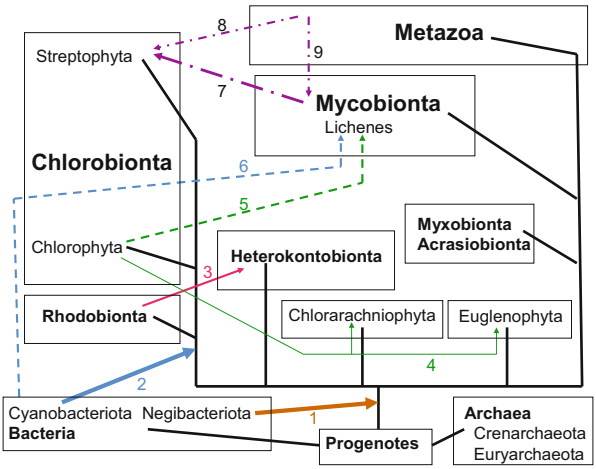

Fig. 10 Interactive relationships between different phyla of organisms. Primary endocytobiosis: (1) gain of mitochondria, (2) gain of plastids. Secondary endocytobiosis: (3, 4) gain of complex plastids. Ectosymbiosis of fungi and algae: (5) with Chlorophyta, (6) with Cyanobacteria. Symbiosis (Ecto- and Endo-) of fungi and plants: (7) mycorrhiza. Interactions between animals and plants: (8) pollination and dispersal of diaspores, (9) symbiosis of animals and fungi. After Bresinsky et al. 2008; complemented

of the "Strasburger" in a team of four authors contributing to the very first editions. One of the later succeeding authors was P. Sitte who together with his co-workers presented convincing observations on the validity of the endosymbiontic theory (Hansmann et al. 1985; Eschbach et al. 1991; Sitte 1993; Maier and Sitte 1994).

A most remarkable scientific result being integrated in academic teaching of our days is the insight: In the course of evolution the diverging phylogenetic lines are interactively and integratively reunited from time to time. The linkage of different groups of organisms (prokaryotes, animals, plants, fungi) into a network, even if phylogenetically quite distant to each other, has essentially promoted the evolution of organic life. Cyanobacteriota (blue-green algae), Rhodobionta (red algae) and Chlorobionta (green plants with the main groups Chlorophyta and Streptophyta) are involved in various interactions as donators of photosynthesis (Fig. 10: 2, 3, 4, 5, 6) or partly also (Chlorobionta) as users of properties developed by other organisms (Metazoa, Mycobionta) to overcome spatial barriers and to improve uptake of mineral nutrients (Fig. 10: 7, 8). With this review, considering also my past life behind as a scientist, I hope that I could give some insight into some of these aspects. Cooperation is substantial for life in its diverse branches and manifestations.

Acknowledgements Special gratitude has to be expressed to Prof. Dr. Ulrich Lüttge, Darmstadt, and to Prof. Dr. Dr. h. c. Wolfgang Steglich, München for encouraging me to write this paper and for the support generously provided. Ulrich Lüttge and I were not only visiting the same University but also the same class in school, not being aware of our future profession within the identical field of science. How could we know at that time that both of us would be separately engaged in the

writing of somewhat competing text books in Botany for the use at Universities? Also Wolfgang Steglich and I, both of us being interested and working on pigments of fungi, are friends for more than 40 years. I always could rely on his profound knowledge and on his competence as an outstanding chemist. Prof. Dr. Jürgen Heinze, a Zoologist, working on ants, and Prof. Dr. Peter Poschlod, both in Regensburg, kindly read the draft of this paper and offered me valuable suggestions. My wife Dr. B. Wittmann-Bresinsky did not only revise the wording of this paper but also contributed results of her own research which were incorporated here. Without the cooperation of my colleagues, co-workers and graduate students it would not have been possible to get knowledge on the details discussed here and which made life so interesting to me. It is not possible to mention all of them by name. A tight affiliation of similar interests and of personal understanding had been grown over the time of common work at the University of Regensburg to Prof. Dr. Peter Schönfelder, Dr. Helmut Besl, Prof. Dr. Michael Fischer, Prof. Dr. Hans-Peter Molitoris and to many other persons. To all of them, regardless whether mentioned by name or not, I am greatly indebted. Last but not least, commemorating my deceased teachers, I have to bow myself in respect and gratitude.

Curriculum Vitae

1935: Andreas Bresinsky was born in Tallinn/Reval, Estonia, as a child of German-Baltic parents.

1939: Resettlement in Poland as a result of the turmoil caused by World War II.

1944: Escape from the invading Soviet Army to Pomerania, Rostock and Lübeck.

1946: Removal to Augsburg in Bavaria.

1954: Graduation from the Gymnasium in Augsburg (present name of the school is Holbein-Gymnasium).

1954: Student at the Ludwig-Maximilians-University in Munich with the subjects biology, chemistry and pedology.

1960: Doctor's degree. Scientific assistant at the Botanical Institute of the Technical University (TH) followed by a position as a curator of the collections of fungi and cryptogamic plants at the Bavarian State Collection of Botany (Botanische Staatssammlung) in Munich.

1964: Habilitation at the Botanical Institute of the Ludwig-Maximilians University in Munich.

1967: Visiting associate professor at Purdue University in Lafayette, Indiana, USA.

1968: Return to the Bavarian State Collection of Botany (Botanische Staatssammlung) holding finally the position of the head's deputy. Chairman of the Bavarian Botanical Society (until 1973).

1973: Accepting the call to a full professorship for Botany at the University of Regensburg/Bavaria. Planning and realisation of the Botanical Garden of the University. Chairman of the Botanical Society of Regensburg (founded in 1790

and nowadays the oldest still existing botanical society of the world). Initiation and support of the relocation of the library and the herbarium of the Botanical Society as a permanent loan to the University of Regensburg.

1990: Convenor of Fourth International Mycological Congress in Regensburg.

2000: Retirement; professor emeritus. Honorary chairman of the Botanical Society of Regensburg (since 1999).

2004 and 2008: Guest lecturer at the Estonian University of Life Sciences in Tartu/ Estonia.

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Part II
Genetics

ABA as a Universal Plant Hormone

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Contents

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Abstract Abscisic acid (ABA) is a sesquiterpene known to regulate environmental stress responses in angiosperms, such as water-loss-induced stomatal closure, development of seed desiccation tolerance during maturation, and salt-, desiccation-, and freezing-stress tolerance of vegetative tissues. An ABA-induced increase in stress tolerance is also reported in other land plant lineages, including nonvascular bryophytes that diverged from vascular plants more than 420 million years ago. Thus, it is hypothesized that acquisition of sensing and response mechanisms for ABA by land plant ancestors was critical for invasion of and adaptation to land. Because bryophytes are key organisms in plant evolution, clarification of their ABA-dependent processes is important for understanding land plant evolutionary adaptation. Based on past and current studies on ABA in non-seed plants and phylogenetic analysis of genome information from various plant species, we discuss the evolution of ABA function and biosynthesis, transport, and signaling network pathways as well as calcium signaling because of its importance in ABA signaling in angiosperms. Future directions of ABA research in the evo-devo field are also discussed.

1 Introduction

The plant hormone abscisic acid (ABA) can be found in various species across kingdoms, including bacteria, fungi, and animals, as well as plants. The roles or functions of ABA in these non-plant species are largely unknown; however, recent reports suggest a ubiquitous role of the sesquiterpene in the regulation of physiological events in non-plant species (Takezawa et al. 2011). Even in the plant kingdom, our knowledge about ABA function largely depends on studies in angiosperms, and a fundamental understanding of ABA activity has just begun to develop for non-angiosperm species. In vascular plants, ABA is produced under water-stress conditions in vegetative tissues and controls stomatal closure and gene expression related to dehydration tolerance (Finkelstein and Rock 2002; Rock et al. 2010). It also regulates maturation, acquisition of desiccation tolerance, and germination of seeds (Finkelstein et al. 2002). In addition, ABA has diverse functions involved in negative control of growth and development such as inhibition of lateral root growth and inflorescence formation (Milborrow 1974). Recent progress in molecular studies of ABA function in basal land plants has revealed its ancient origin in water-stress responses in land plants (Fig. 1) (Takezawa et al. 2011). We can readily note that ABA functions evolved in land plants as a precursor to the acquisition of stomata, vascular system, and seeds that are its target tissues in angiosperms. Thus, understanding the physiological roles as well as the biosynthesis and metabolic and signaling pathways of ABA in non-seed plants will yield insights into how the ubiquitous compound became a plant hormone that is essential for the water-stress response in land plants.

Bryophytes such as mosses and liverworts are nonvascular plants that first emerged 480 million years ago and are widespread across the world from tropical rain forests to regions with harsh environmental conditions such as the desert or Antarctic.

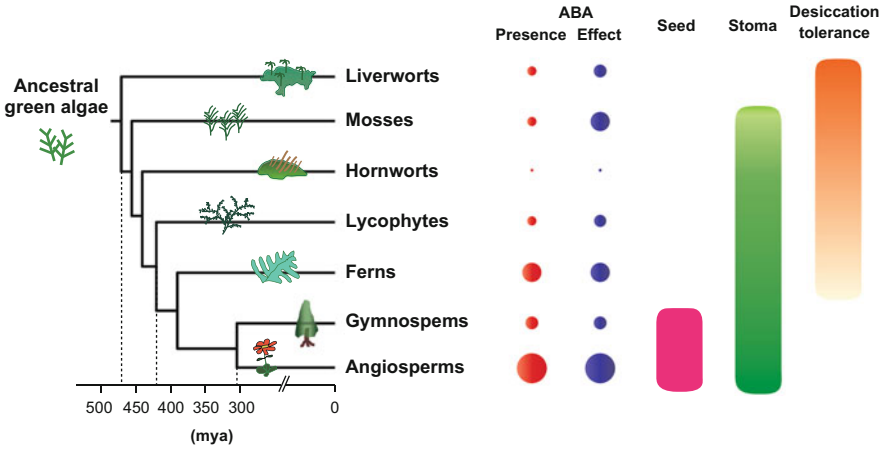

Fig. 1 ABA and land plant evolution. The cladogram shows the evolutionary relationship of land plant groups. Relative effect (*blue*) and endogenous level (*red*) of ABA in each plant groups are shown by dot size. ABA effectiveness in seed development, stomatal regulation, and desiccation tolerance is indicated by the *bar chart*. *Color* strength shows effectiveness

Because they lack water transport and retention tissues, bryophytes engage in a continual equilibrium with the water potentials of the atmosphere (−100 MPa at 28 °C and 50 % relative humidity), a level most modern-day flowering plants could not tolerate. But when watered, they can equilibrate rapidly with surrounding water potential and are fully hydrated without significant damage. This type of desiccation tolerance is likely an ancestral trait of land plants that has been lost from the vegetative tissues of vascular plants during evolution but "re-evolved" in the *Selaginellas* and the *Leptosporangiate* ferns (Oliver et al. 2000, 2005). The mechanisms underlying desiccation tolerance include (1) limiting damage of cellular components caused by severe dehydration stress; (2) maintaining physiological integrity during desiccation; and (3) provoking the cellular repair mechanism upon rehydration (Bewley 1978).

It is critical to find non-angiosperm plants for which tools are available for gene discovery and functional analysis. The moss *Physcomitrella patens* is one of the few such plants (Quatrano et al. 2007). After findings indicating a high frequency of homologous recombination that enables gene targeting (Schaefer and Zryd 1997), this plant has emerged as a model system for analysis of many aspects of plant biology. In 2008, an assembled genome of *P. patens* (487 Mbp) was released from the Joint Genome Institute in the USA (JGI) (Rensing et al. 2008). The predicted gene number is 32,272 which is comparable to findings in angiosperms, and generation of a linkage map with molecular markers is in progress (Kamisugi et al. 2008), which will be a resource for forward genetics. RNA interference (RNAi) (Bezanilla et al. 2003) and artificial microRNAs (Khraiwesh et al. 2008) can be used to downregulate gene expression, an alternative to targeted gene disruption for analysis of gene families in this organism. Taking advantage of this wealth of tools for comparative and functional analysis, molecular dissection of

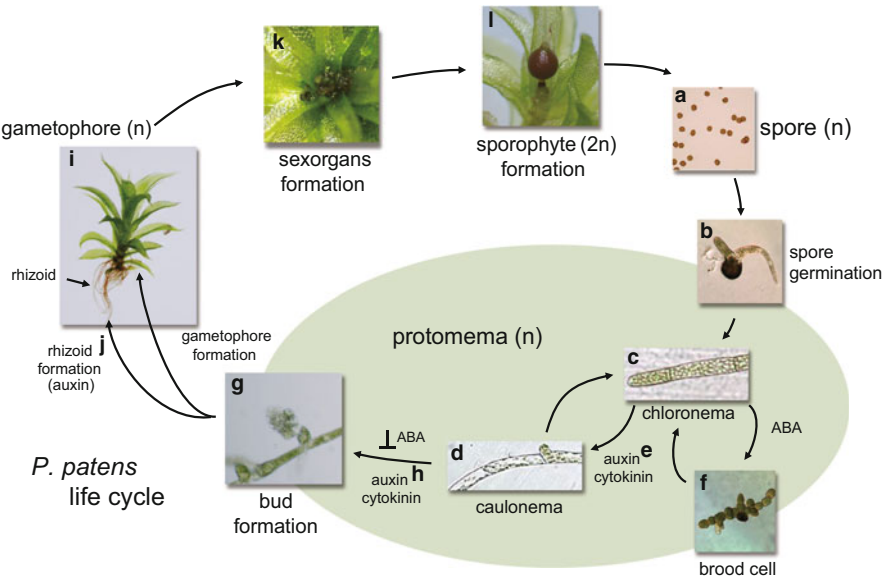

Fig. 2 The life cycle of the moss *Physcomitrella patens*. (**a**) Spores, (**b**) spore germination and generating primary chloronemata, (**c**) chloronema cells, (**d**) caulonema cells, (**e**) caulonema differentiation is regulated by auxin and cytokinin, (**f**) brood cell formation is regulated by ABA, (**g**) young bud, (**h**) auxin and cytokinin induce bud formation, and ABA represses the formation, (**i**) gametophore (leafy shoot) and rhizoid (rootlike tissue), (**j**) auxin induces rhizoid formation, (**k**) a short day and a cold treatment (15 °C) induce sex organ formation, (**k**) after fertilization, the egg cell develops into a sporophyte (diploid) and within its meiosis occurs leading to spore formation. The cycle can be achieved under laboratory conditions in less than 3 months

ABA signaling pathways as well as the biosynthesis pathway in basal land plants is ongoing. For example, recent studies on *P. patens* have revealed developmental and physiological aspects of ABA response in bryophytes (Fig. 2). Gene knockout experiments indicated that desiccation tolerance in the moss involves ABA and the plant-specific transcription factor ABA INSENSITIVE3 (ABI3), which are essential for seed desiccation tolerance of angiosperms (Marella et al. 2006; Khandelwal et al. 2010), suggesting an evolutionarily conserved mechanism for cellular protection from desiccation. Comparative analyses of the common toolkit for ABA functions in land plants will further extend our understanding of the evolution of the signaling machinery that enabled plants to conquer the land.

In this review, we focus on the role of ABA in land plants outside of seed plants including bryophytes, lycophytes, and ferns, and recent progress in the elucidation of an ABA-core toolkit in the model moss *P. patens* and an emerging new model the liverwort *Marchantia polymorpha*. We further discuss the evolution of ABA function, ABA metabolism, and its signaling pathway in relation to calcium signaling that must be related to the innovation of water use efficiency during the evolution of land plants.

2 ABA in Non-seed Plant Lineages

2.1 ABA in Bryophytes

Detection of endogenous ABA in various species of bryophytes by enzyme-linked immunosorbent assays (ELISA) using monoclonal antibodies has been reported. Analysis of endogenous ABA in the desiccation-tolerant liverwort *Exormotheca holstii* has revealed that desiccation of its thalli resulted in a drastic increase in ABA content in comparison with the hydrated thalli (Hellwege et al. 1992). Further analysis indicated that various liverwort species belonging to Marchantiales accumulate endogenous ABA with a large variability in the content ranging from 2 pmol g^{-1}FW to 30 nmol g^{-1}FW (Hartung and Gimmler 1994). In hornworts (*Anthoceros* species), ABA was detected not only in the gametophyte but also in the sporophyte, and stress-induced ABA synthesis was observed (Hartung et al. 1987). In the moss *Funaria hygrometrica*, ABA accumulated during the slow drying process associated with development of desiccation tolerance (Werner et al. 1991). However, these ABA studies require cautious interpretation. Minami et al. (2005) described that the amount of ABA could not be determined precisely by ELISA at least in *P. patens*, probably because of cross-reactivity of the anti-ABA antibody used in the assay with other compounds in crude extracts. Direct measurement of endogenous ABA has been conducted in only limited species of bryophytes. Li et al. (1994) estimated that cultured thalli of *M. polymorpha* contain 4–16 ng of ABA g^{-1}FW by direct measurement by GC-EIMS and GC-NCIMS. In mosses, endogenous ABA has been detected by GC-MS in protonema cells of *P. patens*, and it was found that the cells accumulated more ABA in response to hyperosmotic stress (Minami et al. 2005).

Exogenous application of ABA affects growth and development as well as stress tolerance in bryophytes. In mosses, exogenous ABA inhibits growth of protonema and gametophore (Menon and Lal 1974; Lehnert and Bopp 1983; Chopra and Kapur 1989). ABA also promotes formation of gemma-like structures or spherical "brood cells," which are forms of vegetative reproduction (Chopra and Kapur 1989; Goode et al. 1993; Schnepf and Reinhard 1997). Another well-documented effect of ABA on protonemata is inhibition of gametophore formation. ABA inhibits caulonema's differentiation to gametophore by counteracting with cytokinin that promotes bud formation (Valadon and Mummery 1971; Chopra and Kapur 1989; Christianson 2000). There are also reports indicating that ABA inhibits formation of gametangia for sexual reproduction (Bhatla and Chopra 1981; Chopra and Mehta 1987). In comparison to the studies in mosses, there are a relatively limited number of reports describing the effects of ABA on growth and development of liverworts. ABA inhibits growth of gemmae and thalli (Schwabe and Valio 1970) and formation of gametangia in Marchantiales liverworts (Kumra and Chopra 1986). ABA might play a role in heterophyllous switch of liverworts. Hellwege et al. (1992) reported that exogenous ABA converted thalli of aquatic liverworts *Riccia fluitans* and *Ricciocarpus natans* into the land forms.

In addition to above studies, effects of exogenous ABA on tolerance to desiccation and freezing stress in bryophytes are well described. Application of ABA to cultured protonemata of *F. hygrometrica* induces tolerance to rapid drying (Werner et al. 1991). Pretreatment with ABA of gametophores of *Atrichum androgynum* reduces membrane ion leakage caused by desiccation stress (Beckett 1999). ABA appears to prevent reductions in photosynthesis and non-photochemical quenching caused by desiccation in *A. androgynum* (Mayaba et al. 2001). Effects of ABA on freezing tolerance have been also described. ABA pretreatment of protonemata with cryoprotectants such as proline and sucrose effectively enhanced survival after cryopreservation of moss species (Christianson 1998; Burch and Wilkinson 2002). In *P. patens* protonemata, ABA treatment for one day increases freezing tolerance from −2 °C to −10 °C (Minami et al. 2003). The protonemata accumulate soluble sugars as compatible solutes as well as a number of LEA-like proteins and stress-associated transcripts by ABA treatment (Nagao et al. 2005, 2006).

Exogenous ABA also induces freezing and desiccation tolerance of some liverworts. Pence (1998) showed that ABA pretreatment enhanced survivals after cryopreservation of liverwort species, although the magnitude of the effects of ABA varied among species. ABA effectively increased desiccation tolerance of *Riccia fluitans* while the effect was little on *Plagiochila* sp. and *M. polymorpha* (Pence et al. 2005). The ABA-induced desiccation tolerance appeared to be associated with increased accumulation of total soluble carbohydrates (Pence et al. 2005), though profiles of proteins and gene expression have not been described. These results suggested that effects of ABA on liverworts may vary substantially among different species, but it certainly alters development and desiccation tolerance of some species such as *R. fluitans*. Most of the studies on ABA effects are those on Marchantiales liverworts and very little is known about its effects on other liverwort groups including Jungermanniales.

2.2 ABA in Ferns and Lycophytes

Radioimmunoassays for detection of ABA indicated its distribution in pteridophytes (Weiler 1979). Endogenous ABA has been chemically identified in different species of pteridophytes: protonema of *Anemia phyllitidis* (Cheng and Schraudolf 1974), spores of *Lygodium japonicum* (Yamane et al. 1980), sporophytes of the tree ferns *Cibotium glaucum* and *Dicksonia antarctica* (Yamane et al. 1988), and sporophytes of an aquatic fern *Marsilea drummondii* (Pilate et al. 1989). Recently, detection by more accurate physicochemical methods (GC-MS/MS, GC-SIM, and UPLC-MS/MS) of water-stress-induced foliar ABA levels in ferns (*Pteridium esculentum* and *D. antarctica*) and a lycophyte (*Selaginella kraussiana*) has been reported (Brodribb and McAdam 2011; McAdam and Brodribb 2012). These analyses revealed that foliar ABA levels of ferns and lycophytes are quite low (about 10 ng g^{-1} FW or below) in unstressed control conditions but are markedly increased (about 200–600 ng g^{-1} FW) when exposed to water stress, as observed in seed plants.

Different physiological roles of ABA in growth and environmental responses have been reported.

ABA has a negative effect on growth of gametophyte tissues in ferns. General retardation of growth by ABA of the gametophyte of *L. japonicum* that shows characteristic growth behavior under red and blue light has been reported by Swami and Raghavan (1980). ABA inhibits protonemal elongation, but not spore germination, in *Mohria caffrorum* (Chia and Raghavan 1982). Hickok (1983) showed that ABA stimulates rhizoid production of the *Ceratopteris* gametophyte at concentrations of 10^{-6}–10^{-5} M but is inhibitory at higher concentrations. ABA has been shown to regulate sex determination in *Ceratopteris*. The fern *Ceratopteris* produces either hermaphroditic or male gametophytes, and this developmental fate is determined by the antheridiogen (possibly a gibberellin-like substance) produced by the hermaphroditic gametophytes. Hickok (1983) showed that ABA inhibits antheridiogen-induced formation of male gametophytes. Analysis of ABA-insensitive mutants indicated that these plants develop as males in the presence of antheridiogen and ABA (Warne and Hickok 1991).

ABA also has been shown to control heterophyllous switch in aquatic fern plants. The sporophyte of *Marsilea quadrifolia* produces different types of leaves above and below the water level. It has been shown that its submerged type shoots are modified by ABA, and the development of aerial-type leaves is induced (Liu 1984). Here ABA promoted growth of both leaves and roots but inhibited growth in the internodes. ABA-induced developmental changes returned to the default mode when ABA was removed. A number of ABA-regulated genes, designated *ABRH* for ABA-responsive heterophylly, have been identified from the shoot apex tissues of *M. quadrifolia* (Hsu et al. 2001). Of interest, unnatural R-(−)-ABA has a greater effect on induction of heterophylly and the *ABRH* gene expression than natural S-(+)-ABA, which is possibly due to slow 7′-hydroxylation of R-(−)-ABA for its metabolism (Lin et al. 2005). The heterophyllous switch between aquatic and land forms by ABA has been observed in a wide range of plant species from angiosperms to liverworts (Anderson 1978; Hellwege et al. 1992). Whether or not the mechanisms for these morphogenetic responses by ABA are conserved among different classes of land plants of distantly related taxa is yet to be determined.

The role of ABA in desiccation tolerance of both ferns and lycophytes has been reported. Detached fronds of the desiccation-tolerant fern *Polypodium virginianum* survive slow drying but are severely damaged by drying with silica gel due to rapid water loss. When the fronds are treated with ABA, the amount of water lost is reduced, resulting in better survival after silica gel drying (Reynolds and Bewley 1993a). This process accompanied de novo synthesis of specific proteins (Reynolds and Bewley 1993b). The effect of exogenous ABA on survival after open drying followed by liquid nitrogen storage for cryopreservation of gametophyte tissues of six fern species was examined, and preculture with 10 μM ABA for one week increased survival of the tissues of all species (Pence 2000). Liu et al. reported that ABA functions in desiccation tolerance in the lycophyte. Dehydration treatment of the sporophyte of *Selaginella tamariscina* causes a threefold increase in endogenous

ABA content, associated with upregulation of expression of genes involved in ABA signaling and cellular protection (Liu et al. 2008).

3 Evolution of ABA-Related Genes

With the advance of sequencing technology of DNA, genomic information of various plant species is now available (Phytozome; http://www.phytozome.net). Here we summarize the comparison of numbers of ABA-related genes that are involved in ABA biosynthesis and catabolism and ABA transport and the signaling among plant lineages from green algae to angiosperms (Table 1) and describe insights into the evolution of ABA-related genes in plants. We note that the sequenced plant species are still biased and genomic information from representatives of gymnosperms, ferns, hornworts, and liverworts will be required to complete an evolutionary comparison of ABA-related genes.

3.1 ABA Biosynthesis and Metabolism

The rates of ABA biosynthesis and catabolism are critical to determining the accumulation of ABA as well as the strength of the response. Although a variety of organisms synthesize ABA, the carotenoid pathway in angiosperms is the only defined pathway for ABA biosynthesis (Nambara and Marion-Poll 2005). The Arabidopsis *ABA1* encodes zeaxanthin epoxidase (ZEP), which catalyzes the initiation step of the carotenoid pathway. The cyanobacterium *Synechocystis* genome lacks genes for ABA1 (Yoshida 2005). Our phylogenetic analysis also showed no *ZEP* in the primitive red alga *Cyanidioschyzon merolae* (Takezawa et al. 2011) but identified one *ZEP* ortholog in *C. reinhardtii* (Table 1). The genome of the basal land plant *P. patens* contains a gene set for the carotenoid pathway except for *ABA2*, suggesting that the carotenoid pathway was completed in ancestral land plants. ABA2 appeared only in angiosperms, suggesting the presence of alternative enzymatic pathway to convert xanthoxin to ABA aldehyde. Land plants from bryophytes to angiosperms increase ABA accumulation upon water stresses (Takezawa et al. 2011; McAdam and Brodribb 2012). NCED (9-cis-epoxycarotenoid dioxygenase) catalyzes the rate-limiting step of water-stress-induced ABA biosynthesis in angiosperms (Nambara and Marion-Poll 2005), and *NCED* expression is upregulated by water stress in angiosperms (Xiong and Zhu 2003). The moss *P. patens* is currently the most basal fully sequenced land plant and accumulates ABA upon osmostress (Minami et al. 2005). The *P. patens* genome encodes two *NCED* genes (Table 1) with expression upregulated by dehydration, salinity, and exogenous ABA (Richardt et al. 2010), as observed in vascular plants. These data suggest that water-stress-controlled biosynthesis of ABA was established in the last common ancestor of land plants. Disruption of these genes for the carotenoid pathway of ABA biosynthesis in the basal land plant *P. patens*

Table 1 Comparison of number of abscisic acid (ABA)-related genes among plants

		Green algae	Bryophytes	Lycophytes	Angiosperms	
Function	Gene	*C. reinhardtii*	*P. patens*	*S. moellendorffii*	*O. sativa*	*A. thaliana*
ABA metabolism	ABA1/ZEP	1	1	1	1	1
	ABA4	0	1	1	1	1
	NCEDs	0	2	1	3	5
	ABA2	0	0	0	1	1
	AAO3	0	0	0	0	1
	ABA3	1	1	1	0	1
	BG1	0	0	0	0	1
	CYP707As	0	0	2	3	4
ABA transport	ABCG25	0	0	0	1	1
	ABCG40	0	0	0	10	1
	AIT	0	0	0	1	4
ABA signaling	PYR/PYL/RCARs	0	4	5	11	14
	Group A PP2C	0	2	3	10	9
	Subclass III SnRK2	0	4	2	3	3
	ABI3	0	3	3	1	1
	ABI4	0	0	0	1	1
	ABI5	0	2	4	5	7
	SLAC1	0	4	3	9	5
Ca^{2+}-dependent factor	CIPK/SnRK3	0	7	4	29	25
	CDPK	20	30	10	31	34
	CBL	2	4	3	10	10
	CaM/CML	9	29	26	37	57

The Arabidopsis ABA1, ABA4, NCED3, ABA2, AAO3, ABA3, BG1, CYP707A3, ABCG25, ABCG40, AIT1, PYR, ABI1, SRK2E, ABI3, ABI4, ABI5, SLAC1, CIPKs, CDPKs, CBLs, and CMLs amino acid sequences were used for a BLASTP search to determine the putative orthologs in five species, and the neighbor-joining (NJ) trees were drawn using MEGA5.0. The number of potential orthologs was counted with the NJ-trees. The database used was Phytozome v8.0 (http://www.phytozome.net)

via gene targeting will uncover the significance of the carotenoid pathway for ABA biosynthesis as well as the physiological roles in nonvascular plants.

Several genes involved in ABA catabolism have been identified in Arabidopsis. ABA hydroxylation at the 8′ position to give phaseic acid is the main irreversible catabolic pathway in angiosperms (Nambara and Marion-Poll 2005). The metabolites 7′- and 9′-hydroxy ABA are also reported in some angiosperm species (Zhou et al. 2004). To date, only CYP707A genes for 8′-hydroxylation of ABA have been reported in Arabidopsis (Kushiro et al. 2004), and these genes are involved in drought tolerance (Umezawa et al. 2006) and seed dormancy as well as germination (Okamoto et al. 2006). Another catabolic pathway is conjugation of ABA to glucose-ester (GE), which is subsequently sequestered into vacuoles (Boyer and Zeevaart 1982; Bray and Zeevaart 1985). ABA-GE is reversibly converted to ABA by BETA GLUCOSIDASE (BG) in Arabidopsis. To date, two Arabidopsis BG genes, endoplasmic reticulum-localized AtBG1 and vacuole-localized AtBG2, have been reported to catalyze ABA-GE hydrolysis (Lee et al. 2006; Xu et al. 2012). *AtBG1* disruption results in reduced ABA accumulation in seeds (about 40 % of WT) and in water-stressed leaves (about 80 % of WT). The *atbg1* plants show not only ABA-deficient phenotypes but also a dwarf phenotype with yellow leaves that can be rescued by exogenous ABA (Lee et al. 2006). These data suggest roles for released ABA in the stress response as well as in normal development of Arabidopsis. The authors suggested that this reversible pathway is involved in water-stress-induced rapid ABA accumulation that does not require gene activation. However, a relatively small impact of loss of AtBG1 on ABA accumulation in water-stressed leaves compared to the other phenotypes may suggest roles for AtBG1 beyond ABA release. We also note that no direct evidence of ABA production is provided for the *atbg2* plants that exhibit phenotypes similar to those of the *atbg1* plants (Xu et al. 2012).

An unexpected result was that *P. patens* does not possess genes for any of these enzymes for ABA catabolism. CYP707A genes are present only in vascular plants, and BG1 is present only in angiosperms (Table 1 and Fig. 3). Our preliminary experiment to detect ABA catabolites showed that protonemata of *P. patens* do not accumulate either ABA-GE or 8′-hydroxylation catabolites. Instead, we detected only 9′-hydroxylation catabolites (neoPA) (Sakata et al. unpublished results). *P. patens* actively exports synthesized ABA to the extracellular environment (Minami et al. 2005). The extracellular export system, rather than inactivation and sequestration, is likely to function as the major system to reduce intercellular ABA level in bryophytes. Recently, Okamoto et al. (2011) reported that ABA 9′-hydroxylation is catalyzed by CYP707A as a side reaction in Arabidopsis. Bryophytes may possess other CYPs that catalyze 9′-hydroxylation of ABA as the main reaction.

3.2 ABA Transporters

ABA of angiosperms has been considered as a root-derived signaling molecule that induces physiological changes in shoots in response to dry soil conditions. In fact, expression of Arabidopsis AAO3, AtABA2, and AtANCED3 genes is observed in

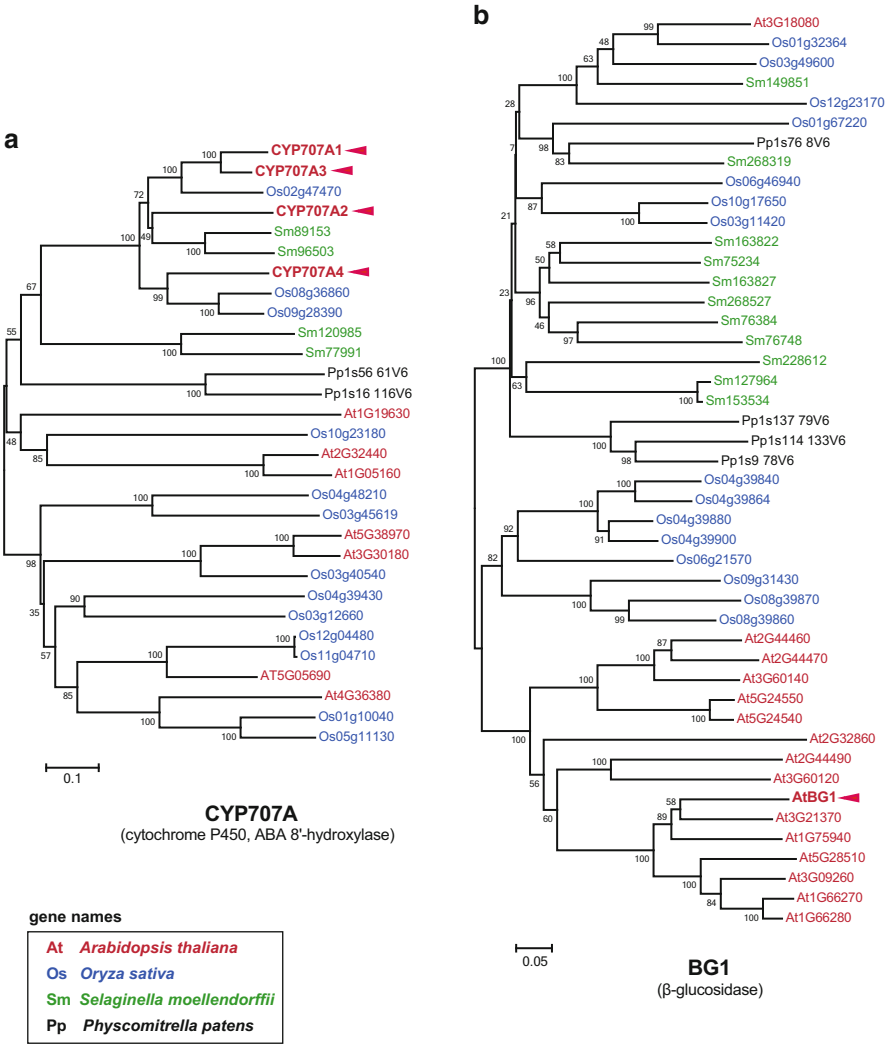

Fig. 3 Phylogenetic relationship among orthologs of the CYP707A family and *BG1 glucosidase*. The phylogenetic tree of CYP707A family (**a**) and *BG1 glucosidases* (**b**) was generated by MEGA5.0 by using the neighbor-joining method. Genes of *A. thaliana*, *O. sativa*, *S. moellendorffii*, and *P. patens* are indicated by *red*, *blue*, *green*, and *black*, respectively. Previously reported Arabidopsis ABA 8′-hydroxylase CYP707A family and *ABA glucosidase* BG1 are indicated by *arrowheads*

vascular tissues (parenchyma cells) in response to water stress (Endo et al. 2008; Koiwai et al. 2004; Seo and Koshiba 2011), indicating that ABA produced in vascular tissues must be delivered to the cells such as guard cells that require water-stress responses. The molecular basis of ABA transportation was only recently clarified. Two independent research groups simultaneously made the breakthrough for ABA transporters using Arabidopsis. Kang et al. (2010) reported that a

pleiotropic drug resistance transporter (PDR)-type ABC transporter PDR12 (AtPDR12/AtABCG40) mediates uptake of ABA into plant cells, and expression of AtPDR12/AtABCG40 in yeast cells and BY2 cells increases ABA uptake. Furthermore, protoplasts derived from *atabcg40* knockout mutant plants showed reduced uptake of ABA. AtPDR12/AtABCG40 is membrane localized and expressed ubiquitously with higher expression in guard cells. The *atabcg40* plants show a delay in gene expression in response to ABA application and are impaired in stress tolerance. Kuromori et al. (2010) isolated an Arabidopsis mutant line with an ABA-sensitive phenotype in the germination and seedling stages from *activator* (*Ac*)/*dissociation* (*Ds*) transposon-tagged mutant collection. The *Ds* element was inserted into the *At1g71960* (*AtABCG25*/*AtWBC26*) gene. Expression of *AtABCG25* was also ubiquitous with the most obvious expression in the hypocotyls, roots, and vascular veins of leaves and enhanced by ABA treatment. Subcellular localization of AtABCG25 was observed at the plasma membrane. ATP-dependent ABA transport activity was observed in membrane vesicles derived from AtABCG25-expressing insect cells, suggesting that AtABCG25 functions as an ABA exporter. Kuromori et al. (2011) also suggested the involvement of AtABCG22—which is closely related to AtABCG25 in the phylogenic tree—in ABA function in guard cells, although direct evidence for ABA transport activity is lacking. More recently, Kanno et al. (2012) identified a novel ABA transporter using an elegant experimental design with Arabidopsis cDNAs capable of inducing interactions between PYR/PYL/RCAR and PP2C in yeasts under low ABA concentrations. The isolated gene, designated ABA-IMPORTING TRANSPORTER (AIT), encoded the low-affinity nitrate transporter NRT1.2, belonging to the NRT1/PTR transporter family, which consists of 53 related sequences (Tsay et al. 2007). AIT1 shows a higher affinity for the natural (+)-ABA than for the synthetic (−)-ABA as its substrate, and is insensitive to an ABA agonist, pyrabactin, indicating specific recognition of ABA structure. We note that AtABCG40 and AtABCG25 had no effect in this assay. AIT1 was localized at the plasma membrane of Arabidopsis cells, and the promoter activity was observed in imbibed seeds and in vascular tissues in cotyledons, true leaves, hypocotyls, roots, and inflorescence stems after germination. The *ait1*/*nrt1.2* plants showed a lower surface temperature in the inflorescence stems than those of the WT due to the excess water loss from open stomata. The finding of an ABA transporter that also transports nitrate partly explains the cross talk between ABA signals and nitrate (Matakiadis et al. 2009).

Of interest, none of these ABA transporters can be found in either the genome of the moss *P. patens* or that of the basal vascular plant *S. moellendorffii* (Table 1 and Fig. 4). We know that *P. patens* exports intercellular cytosolic ABA outside of cells (Minami et al. 2005) and that membrane proteins likely drive this action. Arabidopsis ABA transporters described here might evolve after emergence of seed plants, and there must be other ABA transporters that are yet to be identified.

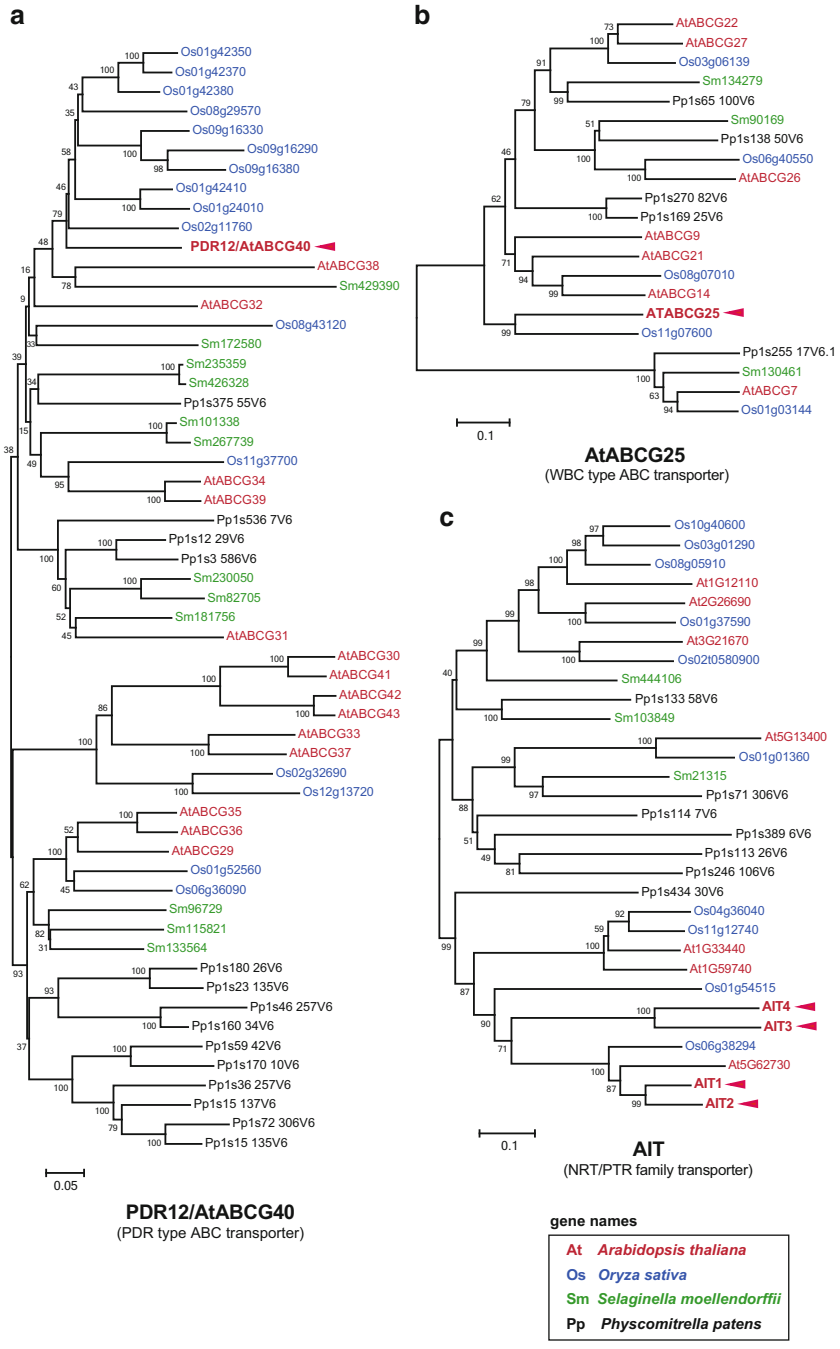

Fig. 4 Phylogenetic relationship among orthologs of ABA transporters. The phylogenetic tree of PDR12/AtABCG40 (**a**), AtABCG25 (**b**), and AITs (**c**) was generated by MEGA5.0 by using the neighbor-joining method. Genes of *A. thaliana, O. sativa, S. moellendorffii*, and *P. patens* are indicated by *red*, *blue*, *green*, and *black*, respectively. Previously reported ABA transporters, PDR12/AtABCG40, AtABCG25, and AITs, are indicated by *arrowheads*

3.3 ABA Signaling Core Components

Compared to enzymes for ABA metabolism and transporters, ABA signaling molecules are well conserved among land plants. Studies in Arabidopsis identified several candidates of ABA receptors including membrane-integrated G-protein-coupled receptors (GPCRs) (Liu et al. 2007), GPCR-type G proteins (Pandey et al. 2009), and the chloroplast-localized Mg chelatase H subunit (Shen et al. 2006); however, their functions as ABA receptors have been questioned or disproven (Risk et al. 2009; Tsuzuki et al. 2011). Only the cytosolic pyrabactin resistance 1/pyrabactin resistance 1-like/regulatory component of ABA receptor (PYR/PYL/ RCAR) (Ma et al. 2009; Park et al. 2009) establishes a robust link to central components of the ABA signaling pathway: plant-specific SNF-related protein kinases (SnRK2s) as positive regulators and ABI1-related type-2C protein phosphatases (PP2Cs) as negative regulators (Takezawa et al. 2011; Umezawa et al. 2010). The three ABA-core components are considered to form an ABA receptor complex to trigger the ABA signaling cascade. ABA-triggered phosphorylation of the transcription factor (Fujii and Zhu 2009) and S-type anion channel activation (Brandt et al. 2012) by the ABA-core components have been reconstructed in vitro. Moreover, an Arabidopsis sextuple mutant of PYR/PYL/ RCAR genes exhibits extreme ABA insensitivity in almost all known ABA-related responses, as is expected from their roles as ABA receptors (Gonzalez-Guzman et al. 2012). These results indicate that the cytosolic ABA receptors play a major role in sensing the hormone in Arabidopsis. The ABA-core components described here are well conserved only in land plants, although their functions outside angiosperms are yet to be elucidated.

Recent studies on model bryophytes indicate that group A PP2C, a member of the ABA receptor core components, plays a role in ABA responses in basal land plants. Komatsu et al. (2009) reported a disruption of one of two *PpABI1* genes (*PpABI1A* and *PpABI1B*) encoding Group A PP2Cs in *P. patens*. Single disruption of *PpABI1A* by gene targeting causes an ABA-hypersensitive phenotype such as increased stress tolerance and enhanced expression of ABA-inducible genes, suggesting that *PpABI1* also functions as a negative regulator of ABA signaling in *P. patens* (Komatsu et al. 2009). More recently, we carried out homology search for signaling molecules for ABA responses in *M. polymorpha* and isolated the *MpABI1* gene that encodes an ABI1-like PP2C (Tougane et al. 2010). To analyze functions of *MpABI1*, a transient assay system by particle bombardment of cultured cells of *M. polymorpha* was established and used for analysis of ABA-induced gene expression. In this assay, the cells bombarded with the *Em-GUS* construct showed ABA-induced GUS expression, and co-expression of the *MpABI1* with *Em-GUS* resulted in suppression of the ABA-induced GUS expression. Furthermore, transgenic *P. patens* plants stably expressing *MpABI1* showed a reduced sensitivity to osmotic and freezing stress and did not undergo typical morphological changes by ABA treatment (Tougane et al. 2010). These results indicated that MpABI1 functions as a negative regulator of ABA signaling.

3.4 Transcription Factors

To date, many transcription factors have been described that are involved in ABA responses of angiosperms. Among them, ABI3 (Giraudat et al. 1992) and ABI5 (Group A bZIP) (Finkelstein and Lynch 2000) isolated from the analysis of Arabidopsis *ABA-insensitive* (*abi*) mutants (Koornneef et al. 1984) are considered to be central to conducting ABA signaling during seed maturation and germination. Of interest, the two transcription factors arose co-instantaneously with other ABA-related genes (Table 1). The shared feature of the transcription factors is that they target ABA-responsive elements (ABREs), with ACGT-core sequences present in the promoters of many ABA-responsive genes of angiosperms (Marcotte et al. 1989; Carles et al. 2002; Hobo et al. 1999; Vasil et al. 1995; Casaretto and Ho 2003). Knight et al. (1995) found that a wheat ABA-inducible *Em* gene promoter is activated by exogenous ABA in the moss *P. patens*, demonstrating evolutionarily conserved machinery from ABA perception to gene activation between *P. patens* and angiosperms. ABI3, also known as VP1 in maize, is an indispensable transcription factor in concert with ABA that regulates maturation and desiccation tolerance of seeds (Giraudat et al. 1992; McCarty et al. 1989). It was unexpected that ABI3/VP1, known as a seed-specific transcription factor, originated in the ancestral land plants. Marella et al. (2006) first reported the function of ABI3 from the moss *P. patens* (PpABI3). PpABI3 can activate the *Em* promoter in both *P. patens* and barley alleurone cells, and partially complements the phenotypes of Arabidopsis *abi3-6* mutant plants. These findings indicate an evolutionarily conserved function of ABI3 in land plants. Sakata et al. (2010) performed comparative analysis of Arabidopsis ABI3 and PpABI3 regarding the activation of the *Em* promoter in *P. patens*. PpABI3 showed more dependency on the other *cis*-element RY sequence, to which the conserved B3 domain of ABI3 binds (Suzuki et al. 1997), while Arabidopsis ABI3 activated the *Em* promoter either in an ABRE- or RY sequence-dependent manner. The difference might be attributed to less-conserved N-terminal regions (B1 and B2) of PpABI3, which are considered to be involved in interaction with other transcription factors (Nakamura et al. 2001; Zhang et al. 2005). These findings suggested that B3 domain-dependent transcriptional regulation of ABI3 is evolutionarily conserved between the moss and angiosperms, whereas angiosperm ABI3 has evolved to activate ABA-inducible promoters independently from the B3 DNA binding domain. Khandelwal et al. (2010) showed that expression of most of the ABA-inducible genes examined was not affected in *abi3* null *P. patens* plants, suggesting that ABI3 is not an essential component for ABRE-mediated gene expression in *P. patens*, as observed in the ABA response of vegetative tissues of angiosperms.

ABI5 belongs to Group A bZIP, which has 13 genes in Arabidopsis (Jakoby et al. 2002). These bZIPs can be further divided into two subgroups based on the conserved N-terminal domains. Nine bZIPs, including ABI5 and AREB/ABFs (ABA-RESPONSIVE ELEMENT BINDING)/(ABA-RESPONSIVE ELEMENT BINDING FACTOR), contain three N-terminal conserved regions termed C1, C2, and C3, and one C-terminal conserved region designated as C4, whereas the

other four bZIPs lack the C1 domain (Bensmihen et al. 2002). The former group (ABI5/AREB/ABF family) is characterized by being involved in ABA signaling in seed development and germination (ABI5, EEL, DPBF2/AtbZIP67, DPBF4, and AREB3) or vegetative tissues (AREB1/ABF2, AREB2/ABF4, ABF1, and ABF3) (Bensmihen et al. 2005; Finkelstein et al. 2005; Fujita et al. 2005; Yoshida et al. 2010) through binding to the ABRE of the ABA-regulated genes via the bZIP DNA binding domain (Choi et al. 2000; Carles et al. 2002; Uno et al. 2000). In contrast to ABI3, Group A bZip has been reported to be absent in the *P. patens* genome and suggested to have first appeared in the most recent common ancestor of spermatophytes; thus it may be related to seed formation (Correa et al. 2008). However, our phylogenetic analysis identified two genes that encode putative Group A bZIPs (Table 1). This fact suggests that the origin of the Group A bZIP is older than proposed. Functional analysis of these genes in *P. patens* will shed light on elucidation of the core component of the toolkit for ABA response in land plants.

It is also known that different types of bZIP transcription factors recognize the ACGT-containing sequences found in seed-specific gene promoters. Opaque2 (O2), a Group C bZIP in maize, binds to TCCACGTAGA in the 22-kDa α-zein promoter (Schmidt et al. 1992). Orthologous genes from wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) are also reported to play the same roles as O2 in their corresponding species (Albani et al. 1997; Oñate et al. 1999). The Group C bZIP is evolutionarily conserved among land plants (Correa et al. 2008), the Arabidopsis genome encodes four Group C bZIP genes, and bZIP10 and bZIP25 have been characterized in association with ABI3. In contrast to maize O2, bZIP10 and bZIP25 are ubiquitously expressed in Arabidopsis tissues (Lara et al. 2003), suggesting an acquired broader range of function outside seeds. These two bZIP proteins physically interact with ABI3 in yeast cells, and co-expression of AtbZIP10/25 with ABI3 results in a remarkable increase in transactivation of the *At2S1* promoter (Lara et al. 2003). A recent study has indicated that heterodimerization of AtbZIP10/25 with another class of bZIP protein (Group S) is also involved in the ABI3-mediated regulation of seed maturation-specific genes in Arabidopsis (Alonso et al. 2009). Richardt et al. (2010) reported in their microarray analysis that two bZIP genes belonging to Group E and G are ABA inducible in *P. patens*. These observations suggest the possibility for the involvement of other classes of bZIPs in the regulation of ABA responses in basal land plants.

4 Evolutionarily Conserved Function of ABA

4.1 Desiccation Tolerance

The definition of desiccation tolerance (DT) is referred as the ability to survive drying to the absolute water contents less than about 0.1 g H_2O g^{-1} dry matter or to a water potential of less than about −100 MPa (Farrant 2010). Several moss species

such as *Tortula caninervis* are known to survive more than −540 MPa (Oliver et al. 2005). Phylogenetic analyses have suggested that DT in vegetative tissue, as expressed in bryophytes, was lost in the vegetative body of vascular plants during the evolution; instead, reproductive organs such as seeds have acquired the trait (Rensing et al. 2008). To survive severe water deficits, plant cells must maintain physiological integrity during dehydration and repair mechanisms must exist that function upon rehydration (Bewley 1979). Some moss species such as *T. ruralis* show constitutive DT without any preconditioning. *T. ruralis* constitutively accumulates soluble sugars and late embryogenesis abundant proteins (LEAs), both of which are suggested to play a major role in protecting cellular integrity upon desiccation (Charron and Quatrano 2009). The desiccated *T. ruralis* produces a set of mRNAs as well as polypeptides whose synthesis is unique to the rehydrated state (Oliver 1991; Scott and Oliver 1994), suggesting that changes in gene expression are also important for cellular repair mechanisms upon the rehydration phase.

Desiccation tolerance of seeds in angiosperms strongly associates with ABA (Finkelstein et al. 2002). ABA is also implicated in regulation of DT in bryophytes. In contrast to the desiccation-tolerant mosses, several moss species such as *P. patens* and *Funaria hygrometrica* are drought tolerant but not tolerant to rapid drying. Slow desiccation treatment of *F. hygrometrica* leads to loss of almost all of their water after 24 h with an induced level of endogenous ABA of about sixfold and recovery after rehydration. Application of exogenous ABA at greater than 10 μM confers tolerance to rapid drying, and exogenous ABA has no effect on the rate of water loss (Werner et al. 1991). These data suggest that the DT of *F. hygrometrica* is not constitutive but inductive and that ABA plays a key role in the induction. This ABA-induced desiccation tolerance has been further analyzed at the molecular level using the model moss *P. patens*. Without preconditioning *P. patens* does not survive water potentials lower than about −13 MPa; however, 50 μM ABA treatment prior to desiccation increases survival against at least −273 MPa (Koster et al. 2010). Application of several micromolar ABA also establishes tolerance to rapid desiccation by exposing to an atmosphere of about −100 MPa (about 50 % relative humidity) in a laminar flow cabinet for 24 h (Khandelwal et al. 2010). Osmostress also induces accumulation of endogenous ABA in *P. patens* (Minami et al. 2005). These facts also indicate a role for ABA in induction of DT in *P. patens*. Furthermore, exogenous ABA induces accumulation of boiling-soluble proteins in *P. patens*, a characteristic feature of hydrophilic LEA proteins which specifically accumulate in developing seeds of angiosperms (Knight et al. 1995). ABA treatment of *P. patens* also induces accumulation of soluble sugars such as sucrose (Oldenhof et al. 2006; Nagao et al. 2006) and a trisaccharide theanderose (Nagao et al. 2006). Oldenhof et al. (2006) reported that exogenous ABA preserves membrane phase behavior in the dried state and that the membranes do not undergo a gel to liquid membrane phase transition during dry–rehydration process. It is likely that ABA functions to elicit cellular protection mechanisms upon the rehydration phase to regain integrity of membranes.

The Arabidopsis *abi3* mutant lacks expression of a set of seed-specific genes including *LEA* and desiccation tolerance of seeds (Nambara et al. 1995; Marella et al.

2006), indicating that ABA- and ABI3-mediated gene expression is key for desiccation tolerance of seeds. The ABA- and ABI3-mediated regulation of LEA gene expression is evolutionarily conserved between *P. patens* and Arabidopsis (Marella et al. 2006). Moreover, disruption of ABI3 results in loss of ABA-induced DT of *P. patens* (Khandelwal et al. 2010). These data collectively suggest that the mechanism that confers desiccation tolerance of plant cells was established in the last common ancestor of bryophytes and angiosperms. During evolution, land plants acquired a way to execute the induction of desiccation tolerance in an ABA-dependent manner, and the system independently has been used to provide desiccation tolerance of vegetative tissues of bryophytes and angiosperms seeds.

4.2 Low Temperature Responses

Overwintering plants in the temperate and sub-frigid zones of earth in general can acclimate to low temperatures, and these plants develop freezing tolerance in response to pre-exposure to nonfreezing low temperature. It has been suggested that ABA plays a role in this cold acclimation process. Endogenous ABA levels increase during cold acclimation (Daie and Campbell 1981; Lalk and Dorffling 1985; Smoleńska-Sym et al. 1995; Jouve et al. 2000), and exogenous ABA treatment at ambient temperatures increases freezing tolerance (Chen and Gusta 1983). Analysis of transcripts has indicated that ABA treatment increases expression of a number of stress-responsive genes that are also induced by cold (Zeevaart and Creelman 1988). These results suggest that the endogenous ABA accumulated in response to low temperature exposure plays a role in enhanced freezing tolerance. Accordingly, analysis of cold-induced genes of Arabidopsis has revealed the frequent occurrence of ABRE in the promoters (Yamaguchi-Shinozaki and Shinozaki 1994; Seki et al. 2002).

Despite these facts, the role of ABA in cold acclimation has yet to be conclusively established (Gusta et al. 2005). Studies on ABA-deficient *aba1* and ABA-insensitive *abi1-1* of Arabidopsis have indicated that the contribution of ABA to cold acclimation might be limited. Both ABA-deficient *aba1* and ABA-insensitive *abi1-1* mutants showed accumulation of cold responsive (COR) transcripts by low temperature treatment (Gilmour and Thomashow 1991). In addition, cold-induced gene expression is mediated by CBF/DREB transcription factors that bind to the distinct *cis*-element C-repeat, also known as the dehydration-responsive element (DRE), independent of ABA (Baker et al. 1994). These results suggest that endogenous ABA has little or no role in the induction of cold tolerance. However, the issue is not simple because the C-repeat/DRE can serve as a coupling element (CE) with ABRE; thus, the C-repeat/DRE contributes to the ABA-induced gene expression when present with ABRE (Narusaka et al. 2003). In addition, expression of genes for CBFs/DREBs is induced by ABA (Knight et al. 2004). In contrast to molecular studies of cold responses in Arabidopsis, such studies on other plant species with various cold tolerances are limited. Recent global promoter analyses indicate that both ABRE and C-repeat/DRE are conserved in cold-induced promoters of Arabidopsis and soybean. ABRE

is also conserved in the cold-induced promoter of rice, but the C-repeat/DRE is not (Maruyama et al. 2012). These results indicate that the mechanism for cold-induced gene expression might vary even among angiosperm species, though implicating the presence of evolutionarily conserved *cis*-promoter elements regulated by ABA.

Even nonvascular plants can undergo cold acclimation. Moss species growing in their natural habitat have a higher freezing tolerance in winter than in summer (Rütten and Santarius 1992). Laboratory experiments using *P. patens* have indicated that its protonemata and gametophore develop freezing tolerance in response to exposure to nonfreezing cold temperatures for several days (Minami et al. 2005; Sun et al. 2007). The increase in freezing tolerance accompanies an increase in various LEA-like transcripts, which are also inducible by ABA treatment. *P. patens* genes, which are remarkably induced by cold acclimation, contain putative ABREs but not C-repeats/DRE in the promoter, indicating a possible role of evolutionarily conserved *cis*-elements in cold-induced gene expression (Cuming et al. 2007; Bhyan et al. 2012). In contrast to these facts, analysis of endogenous ABA levels has suggested that obvious increases in ABA levels are not observed during up to one week of cold acclimation (Minami et al. 2005). Thus, the contribution of endogenous ABA in cold acclimation of moss is not yet conclusive.

Our recent results using ABA-insensitive AR7 lines of *P. patens* indicate that the ABA signaling process is necessary for cold responses in mosses. The AR7 mutant that has been isolated by ultraviolet mutagenesis and the D2-1 line that overexpresses the catalytic domain of Group A PP2C show an ABA-insensitive growth and cannot develop freezing and desiccation tolerance. Of interest, these lines did not acclimate to low temperature to enhance freezing tolerance, with reduced accumulation of stress-associated transcripts (Bhyan et al. 2012). These results indicate that cold and ABA signal transduction share a common mechanism regulated by reversible phosphorylation. Identification of the molecule under such regulation as well as transcription factors responsible for the cold-induced gene expression would clarify how ABA signaling molecules modify the cold-response pathway.

4.3 Stomatal Closure

Stomata are widely distributed among land plants except liverworts, indicating that the epidermal apparatus evolved after the separation of liverworts and mosses from a common ancestor more than 400 million years ago (Fig. 1). The role of ABA in seed plant stomatal closure is well documented (Schroeder et al. 2001; Rock et al. 2010; Mori and Murata 2011). Water-stressed seed plants increase accumulation of ABA, which effectively closes the stomatal aperture by reducing the turgor pressure of guard cells. This physiological event is considered as one of the most important functions of ABA to protect plants from water deficiency. However, ABA signaling can be found in liverworts (Tougane et al. 2010), suggesting that the role of ABA in stomatal closure is an evolutionarily acquired feature. It is still a matter of debate as to when land plants acquired the ABA-dependent closure system of the stomatal aperture in their evolutionary history; one group has reported that ABA

involvement in regulating plant water loss and hydration through stomata is found only in seed plants, but another group has suggested that ABA influences the guard cell aperture of the basal vascular plants. Brodribb and McAdam (2011) examined the ABA response of the stomata of lycophytes and ferns by adding a high concentration of ABA to the transpiration stream. Even with the high concentration of foliar ABA (7,000 ng g^{-1} FW), stomata of fern (*Pteridium esculentum*) and lycophyte (*Lycopodium deuterodensum*) species lacked the closure response, although angiosperm (*Helianthus annuus*) and conifer (*Callitris rhomboidea*) species showed rapid stomatal closure at leaf ABA levels of 1,500–2,000 ng g^{-1} FW. Moreover, they showed a strong correlation between leaf water content and stomatal aperture in lycophytes and ferns. These authors suggested that stomatal closure of lycophytes and ferns is passively controlled and insensitive to ABA and that active metabolic control of stomatal closure evolved after the divergence of ferns about 360 million years ago. However, this hypothesis has been challenged (Ruszala et al. 2011). They examined stomatal responses of the lycophyte *Sellaginella uncinata* to ABA as well as CO_2 in comparison with Arabidopsis. Exogenous ABA from 0 to 25 μM inhibited the light-induced opening of pre-closed stomata and promoted stomatal closure of pre-opened stomata dose dependently, although McAdam and Brodribb (2012) specifically examined this finding and could not repeat it (see below). This ABA effect was blocked in the presence of EGTA and verapamil, which inhibit ABA-induced calcium transient in angiosperm guard cells. ABA also induced an increase of reactive oxygen species (ROS) in *S. uncinata*, the intracellular second messenger of the Ca^{2+} transient in angiosperms guard cells. These authors further demonstrated that the *S. uncinata* gene encoding an SnRK2 similar to Arabidopsis OST1 could complement the Arabidopsis *ost1* mutant phenotype. The stomata of *S. uncinata* also exhibited CO_2-induced stomatal closure as observed in seed plants. These data suggested that evolutionarily conserved mechanisms operate stomatal closure between *S. uncinata* and angiosperms. According to this report, active stomatal control emerged in ancestral vascular plants about 420 million years ago. However, an evolutionarily conserved function of OST1-like SnRK2 may not necessarily be related to the conserved mechanism of stomatal closure because this type of kinase can be found in the liverwort *M. polymorpha*, which lacks stomata (data not shown).

The conflict between the reports may be attributed to the method or plant materials they employed; however, guard cells of ferns and lycophytes might possess a signaling pathway that can be activated by ABA. In fact, a set of genes that encode signaling components and biosynthesis enzymes for ABA in angiosperms is evolutionarily conserved among land plants including the basal land plant bryophytes that diverged from vascular plants 420 million years ago. It is intriguing that the known ABA transporter genes can be found only in seed plants (Table 1). Acquisition of these genes in seed plants might contribute to the rapid reaction of the stomatal aperture in response to environmental water conditions. As mentioned first in this section, liverworts, which represent the oldest extant lineage of land plants, lack stomata but possess the ABA signaling pathway, indicating that ABA evolved as a phytohormone to regulate physiological aspects other than

stomatal control. A key question is when endogenous ABA acquired a role in controlling the turgor pressure of guard cells. McAdam and Brodribb (2012) further investigated the function of water-stress-induced endogenous ABA in stomatal closure of the lycophyte (*S. kraussiana*) and ferns (*P. esculentum* and *Dicksonia antarctica*) in comparison with seed plants (*Pisum sativum*, *P. radiata*, and *Callitris rhomboidea*). They exposed plants to water stress that induced stomatal closure and increase of endogenous ABA levels in all plant species examined. Rapid rehydration of excised, drought-treated leaves quickly restored their water potential while maintaining high concentration of ABA. This manipulation clearly discriminated the stomatal response of seed plants from that of lycophyte and ferns. Stomata of the lycophyte and ferns rapidly opened their stomata upon rehydration while stomata of seed plants failed to reopen upon rehydration probably because of a high level of foliar ABA. These authors suggested that endogenous ABA is not involved in the regulation of stomatal closure of lycophytes and ferns. Under these circumstances, we need more studies to draw any conclusions about the role of endogenous ABA in stomatal closure in the basal vascular plants. Ultimately, elucidation of the role of ABA in basal vascular plants will require genetic modification of ABA signaling or biosynthetic pathways, which is still difficult to perform in these species at this stage.

The basal land plants bryophytes occupy an important place in elucidating the evolution of stomatal control in land plants. However, the physiology of stomata in basal land plants is poorly understood. Among bryophytes, stomata are found in mosses and hornworts but not in liverworts. The current understanding is that stomata arose only once during land plant evolution after the divergence of liverworts from other bryophytes (Vatén and Bergmann 2012). Stomata occur in the sporophytes of mosses and hornworts, and those of *Sphagnum*, sister to other mosses, are considered to function in facilitating sporophyte desiccation (Duckett et al. 2009). Stomata from the hornwort *Anthoceros* species have been reported to close in response to exogenous ABA (Hartung et al. 1987). Lucas and Renzaglia (2002), however, examined the behavior of stomata from several hornworts including *A. caucasicus* and observed no responses to light/dark conditions and ABA (Lucas and Renzaglia 2002). These authors suggested that the stomata of hornworts function in providing a passageway for gas exchange. Further analyses will be required to evaluate these reports. Stomata of mosses of the Funariaceae including *P. patens* are located only in the ring around the base of the diploid sporophyte structure and have been confirmed to close in response to a light-to-dark shift, atmospheric CO_2 levels, and exogenous ABA (Chater et al. 2011). The fungal toxin fusicoccin, which activates the H^+-ATPase pump essential for turgor-driven stomatal opening in angiosperms, also induced stomatal opening of *P. patens* and *F. hygrometrica*. Responsiveness of the ABA-inducible *PpLEA1* promoter was observed at the base of the sporophyte around the stomatal ring, suggesting activation of ABA signaling in this region. They also demonstrated that a gene encoding *Physcomitrella* SnRK2 (PpOST1) functionally complements the phenotype of the Arabidopsis *ost1* mutant and disruption of *PpOST1* led to attenuation of ABA-induced stomatal closure of *P. patens*. Molecular genetic analysis suggests that ABA can change the stomatal aperture in *P. patens* and *F. hygrometrica* through a

functionally conserved OST1-like SnRK2; however, this ABA response may be nonadaptive because the stomata of the bryophyte sporophyte are considered to act to divert water and solute from the parental gametophytes or in the desiccation of sporophytes (Ligrone et al. 2012a, b).

5 Role of Cytosolic Calcium and Calcium-Binding Proteins in ABA Signaling

It has been discussed that calcium, a ubiquitous second messenger, plays a role in ABA-induced signaling processes in plants. Calcium concentrations in cytosol ($[Ca^{2+}]_{cyt}$) maintained at sub-micromolar levels can be increased in response to extracellular signals through opening of plasma membrane and endomembrane-located calcium channels. The generated calcium signals are decoded by various types of calcium-binding proteins (CaBPs) that undergo drastic conformational changes upon calcium binding to a calcium-binding loop called the EF hand. Many biotic and abiotic stress responses are thought to be mediated by $[Ca^{2+}]_{cyt}$ and CaBPs by regulation of functions of intracellular signaling molecules as well as gene expression driven by transcription factors (Trewavas and Malhó 1998; Kudla et al. 2010; Reddy et al. 2011).

More than 200 EF-hand CaBPs are encoded in the Arabidopsis genome (Day et al. 2002). A major group of these are low molecular weight CaBPs which do not have domains with catalytic functions but interact with other enzymes to modulate their functions; the other groups have a distinct catalytic domain with activity regulated by the calcium-binding domain within the same polypeptide. The former group includes calmodulin (CaM), CaM-like proteins (CMLs), and calcineurin B-like proteins (CBLs), and the representative of the latter is calcium-dependent protein kinases (CDPKs).

5.1 CDPKs

The role of calcium in ABA responses has been demonstrated in guard cells of stomata for their stress-induced closure process (Schroeder and Hagiwara 1990; Leckie et al. 1998). During stomatal closure, ABA inhibits plasma membrane hyperpolarization and influx of potassium ion to cytosol, while facilitating the release of potassium by triggering membrane depolarization. The influx of calcium to cytosol has been characterized as one of the initial events occurring during the stomatal ABA response. The initial $[Ca^{2+}]_{cyt}$ elevations serially induce further Ca^{2+} release from the vacuoles (McAinsh et al. 1995), thus generating specific oscillation patterns of cytosolic calcium distribution (McAinsh et al. 1990, 1995). The calcium oscillation induced by ABA causes depolarization of plasma membrane, facilitated by S-type anion currents in guard cells leading to opening of the outward rectifier potassium channel (Pei et al. 1997; Siegel et al. 2009).

In Arabidopsis, a central guard cell S-type anion channel (SLAC1) has been identified as a key regulator facilitating calcium-induced membrane depolarization during stomatal closure (Vahisalu et al. 2008). Recent reports suggest the involvement of CDPKs in the regulation of SLAC1 activity. CDPKs, belonging to the SNF1 protein kinase family, have a serine–threonine protein kinase domain fused to a CaM-like domain with EF hands. Binding of calcium to the EF-hand motifs results in release of autoinhibition of the catalytic domain and makes the enzyme active (Harmon et al. 2000). An Arabidopsis *atcpk3*/*atcpk6* double mutant that shows impaired ABA and Ca^{2+}-induced stomatal closure has reduced S-type anion channel activity (Mori et al. 2006). ABA-induced activation of SLAC1 channels has been reconstituted in *Xenopus* oocytes using the ABA receptor PYR1 and Group A PP2Cs with either CPK6 or OST1 (Brandt et al. 2012). These studies indicate that for stomatal regulation, CDPK can activate the SLAC1 channel in parallel with activation by the subclass III SnRK.

CDPKs appear to play a role in ABA-induced gene expression. Mutations of two Arabidopsis CDPKs, *AtCPK4* and *AtCPK11*, result in ABA-insensitive phenotypes in seed germination and seedling growth, and the double mutants have stronger ABA insensitivity than the single mutants (Zhu et al. 2007), indicating that the two CDPKs function as positive regulators of ABA signaling. Group A bZIPs (ABF1, ABF2, ABF3, and ABF4), responsible for ABA-induced gene expression in the vegetative tissues of Arabidopsis, are activated by ABA-induced phosphorylation. In vitro, AtCPK32 interacts with and phosphorylates the ABA-induced transcription factor ABF4, which directly binds ABA-inducible promoters. The CPK4 and CPK11 kinases both phosphorylate two ABA-responsive transcription factors, ABF1 and ABF4 (Zhu et al. 2007). Lynch et al. (2012) reported that CPK11 very efficiently phosphorylates the N-terminal halves of both ABF1 and ABF3 but not of ABF2 and ABF4. ABFs are also direct targets of phosphorylation by the subclass III SnRK, and the ABA-activated phosphorylation of the ABF2 fragment is completely impaired in the *srk2d*/*e*/*i* triple mutant in vitro. The ABA insensitivity of the *cpk4 cpk11* double mutant is also limited compared to that of the *srk2d*/*e*/*i* triple mutant; thus, CDPKs might be partially involved in ABA signaling through the phosphorylation of specific ABFs in parallel with or downstream of SnRK2s, although how ABA activates the kinases is yet to be elucidated.

The Arabidopsis genome encodes 34 CDPKs, and these kinases are also conserved in non-seed plants as well as in green algae, the latter of which lack ABA-related genes (Table 1 and Fig. 5a). The number of CDPK genes appears to be unrelated to the evolution of green plants, suggesting their fundamental roles in plant life, although their roles in the non-seed plants are largely unknown.

5.2 Other CaBPs

CBLs with sequence similarity to mammalian calcineurin B have targeted a family of protein kinases referred to as CIPKs (CBL-interacting protein kinases)

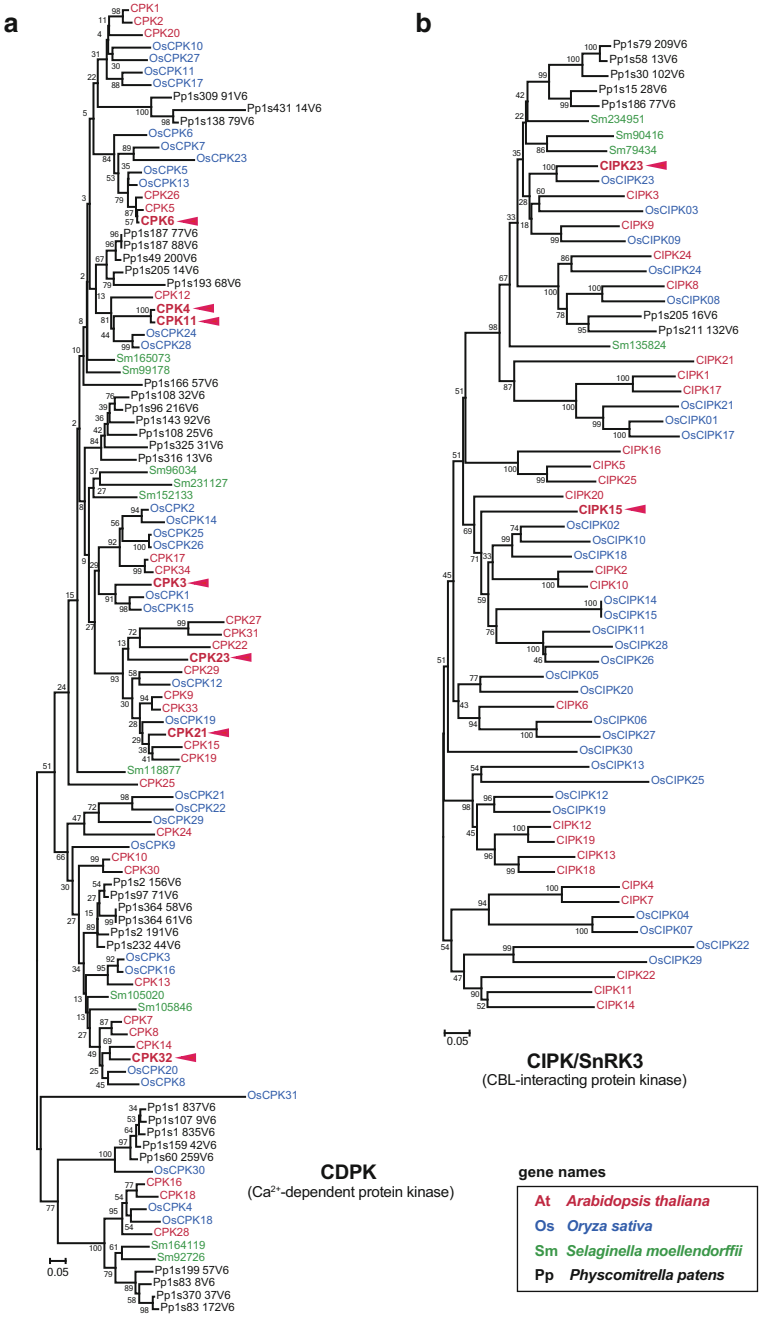

Fig. 5 (continued)

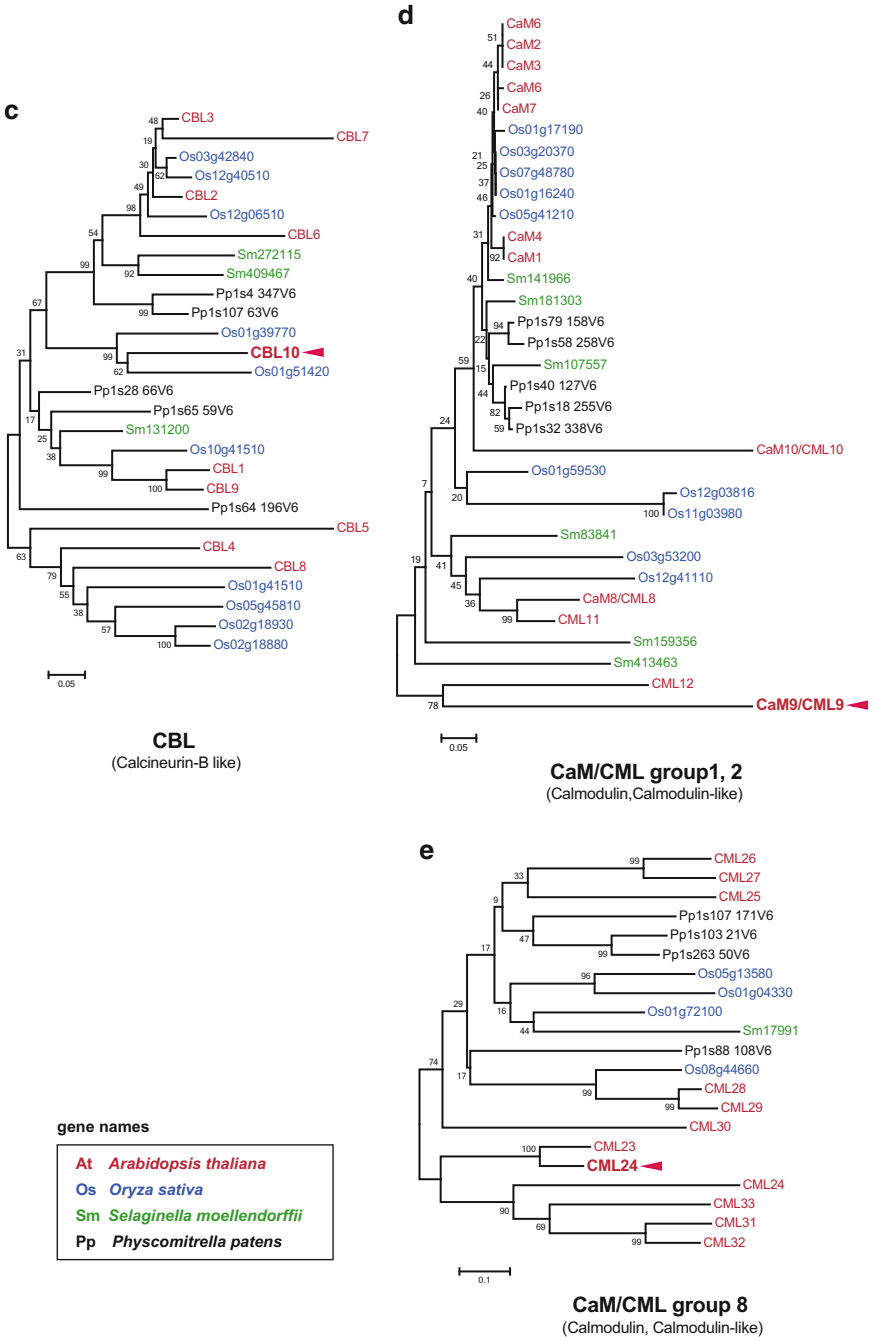

Fig. 5 Phylogenetic relationship among orthologs of Ca^{2+}-dependent components. The phylogenetic tree of CDPK (**a**), CIPK (**b**), CBL (**c**), and CML (**d**, **e**) was generated by MEGA5.0 by using the neighbor-joining method. Genes of *A. thaliana*, *O. sativa*, *S. moellendorffii*, and *P. patens* are indicated

(Luan 2009; Kudla et al. 2010). By genetic analysis of salt-overly-sensitive mutants of Arabidopsis, *SOS3* (*AtCBL4*) and *SOS2* (*AtCIPK24*) have been identified as loci for salt stress sensing (Halfter et al. 2000). Sequencing of the Arabidopsis genome has revealed 25 CIPKs targeted by 10 CBLs (Table 1 and Fig. 5b, c). These proteins have diverse functions in plant responses to environmental stresses as well as to light, hormone, and sugars (Batistic et al. 2010). Expression of Arabidopsis *AtCIPK3* is responsive to exogenous ABA and cold, salinity, and wounding. Disruption of *AtCIPK3* and its interacting *AtCBL9* results in an ABA-hypersensitive phenotype in seed germination, indicating that the AtCIKP3/AtCBL9 is a negative regulator of ABA signaling (Kim et al. 2003; Pandey et al. 2004, 2008). In contrast, transgenic plants lacking AtCBL1 are sensitive to drought, cold, and salt stress independent of ABA, indicating its role in positive regulation of stress responses (Albrecht et al. 2003; Cheong et al. 2003). Both AtCBL1 and AtCBL9 can target to AtCIPK1, and ABA-dependent and -independent signaling processes are regulated (D'Angelo et al. 2006). In rice, Xiang et al. (2007) reported that among 20 CIPK genes (OsCIPKs) tested, the expression of the majority of CIPKs was found to be induced by ABA (16 genes), drought (15 genes), salt (12 genes), and cold (3 genes). Transgenic plants overexpressing the transgenes *OsCIPK03*, *OsCIPK12*, and *OsCIPK15* showed improved stress tolerance with increased proline contents compared to wild type (Xiang et al. 2007). These results suggested that CBL/CIPKs have functions in ABA signaling and stress tolerance in angiosperm species. CBLs are found not only in angiosperms but also in *P. patens* and green algae (Table 1 and Fig. 5b, c). On the other hand, CIPK appears to be unique in land plants, although angiosperms have a greater number of CBL/CIPKs compared to non-seed plants. This pattern indicates that the numbers increased with increasing morphological and physiological complexity in land plant evolution.

CaM targets multiple proteins through calcium-dependent binding to basic amphipathic amino acid stretches in the regulatory domain. Protein kinases are among important targets of CaM, based on information obtained in animal studies of calcium/CaM-dependent protein kinases (Hanson and Schulman 1992). A screen using ^{35}S-labeled CaM identified a receptor-like kinase (RLK) CRCK1 from Arabidopsis. The expression of *CRCK1* was increased in response to cold and salt stress, H_2O_2, and exogenous ABA (Yang et al. 2004). Although targets of CaM-binding RLKs have not been identified, these RLKs might regulate the stress-signaling pathway by affecting the MAP kinase cascade (Yang et al. 2010a, b). In addition, transcriptional activators can be targets of CaM for the control of stress-induced gene expression mediated by calcium. Overexpression of GmCaM4, a divergent CaM isoform that specifically binds to the MYB2 transcription factor, resulted in

Fig. 5 (continued) by *red*, *blue*, *green*, and *black*, respectively. Previously reported ABA signaling-related components, CPK3, CPK4, CPK6, CPK11, CPK21, CPK23, CPK32, SLAC1, CIPK15, CIPK23, CBL10, CML9, and CML24, are indicated by *arrowheads*. CML groups according to the phylogenetic analysis of Arabidopsis CaM and CML (McCormack et al. 2005)

salt stress tolerance and increased expression of *P5CS1*, *ADH1*, and *RD22* (Yoo et al. 2005). MYB2 is known as a transcription factor induced by dehydration, salt, and ABA, and its overexpression resulted in enhanced ABA sensitivity (Urao et al. 1993); thus, CaM interaction adds another regulatory mechanism of calcium. CaM also interacts with other transcription factors such as TGA3, GT-2-like1 (GTL1), WRKY, and NAC domain transcription factors as well as ABF2/AREB1, potentially being a target of CaM regulation (Popescu et al. 2007a, b) (for review, see Kim et al. 2009; Galon et al. 2010). These transcription factors might also play a role in calcium-dependent modification of the ABA signaling pathway.

The Arabidopsis genome encodes 50 CMLs according to classification by (Day et al. 2002). Physiological functions of most CMLs have not been identified, but some CMLs are likely to be regulating ABA signaling. In Arabidopsis, *AtCML24* transcripts were induced by multiple environmental signals. *AtCML24*-underexpressing transgenics are less sensitive to ABA in germinating seeds and seedlings (Delk et al. 2005). Another CML, AtCML9, is rapidly induced by abiotic stress and ABA in young seedlings, and in the *cml9* knockout mutants showed a hypersensitive response to ABA with enhanced tolerance to salt and drought stress (Magnan et al. 2008). Targets of most CMLs, including AtCML9 and AtCML24, are yet to be identified.

5.3 The Role of Calcium in Basal Land Plants

Circumstantial evidence indicates that calcium plays a role in bryophytes' responses to ABA and environmental stresses. Cold-, touch-, and blue/UV-A light-induced increase in cytosolic calcium has been demonstrated in *P. patens*, suggesting that calcium is a ubiquitous intracellular messenger in land plants (Russell et al. 1996, 1998; Tucker et al. 2005). The role of cytosolic calcium in stress responses in mosses has been suggested by the study of the *P. patens PCA1* gene encoding a PIIB-type Ca^{2+}-ATPase whose transcript accumulation is induced by dehydration, NaCl, and ABA. The study indicated that disruption of *PCA1* gene results in elevated cytosolic calcium levels upon salt stress. Furthermore, *PCA1* knockout plants showed reduced transcript levels of *PpCOR47* in response to ABA and salt stress and were more susceptible to salt stress than the wild type (Qudeimat et al. 2008). These results indicate that homeostasis of cytosolic calcium levels is important for ABA and stress sensing and signaling processes. Calcium-binding proteins that sense changes in calcium concentrations in *P. patens* remain to be elucidated. The *P. patens* genome encode 29 CaMs, four CBLs, seven CIPKs, 30 CDPKs, and probably more than two dozen CMLs, which might play roles in ABA and stress responses in mosses (Table 1 and Fig. 5).

Regulation of ABA signaling by $[Ca^{2+}]_{cyt}$, a universal second messenger, might have provided land plants with novel mechanisms to flexibly alter their stress responses, which change moment by moment in the terrestrial environment. Identification of target molecules for regulation by $[Ca^{2+}]_{cyt}$ is key to understanding acquisition of such

mechanisms. Genes of CBLs/CIPKs, CDPKs, and CaM-binding transcription factors are likely to have appeared long before the emergence of ABA-related genes, suggesting that the integration of calcium signaling into ABA signaling might occur during evolution of land plants. Clarification of the role of $[Ca^{2+}]_{cyt}$ in ABA signaling of bryophytes will further extend our knowledge about stress adaptation in land plants.

6 Conclusions and Perspectives

Since pioneering studies in the 1960s, ABA had been held as a plant hormone that regulates water-stress responses mainly in vegetative stomata and reproductive seeds of angiosperms. Recent progress in sequencing technology as well as physiological and molecular analyses of basal land plants has demonstrated the origin of ABA in the last common ancestor of extant land plants. Key issues for future evo-devo studies of ABA include pinpointing the origin of ABA signaling and how this signaling has been modified to establish seed development and stomatal regulation.

To address how ABA signaling evolved during plant evolution, gibberellin (GA) would be a good model. GA signaling observed in angiosperms is evolutionarily conserved in the lycophyte *S. moellendorffii* but not in the bryophyte *P. patens* (Hirano et al. 2007). A recent study suggested that the GA perception system evolved to regulate a preexisting GAMYB-based system for spore and sexual organ development during land plant evolution (Aya et al. 2011). The GA studies indicate that the ABA perception system might have merged during evolution of algae to land plants to regulate an as-yet unknown preexisting system in a water-stress-dependent manner. Charophyceae species are the closest relatives of the green land plants. Although the genome sequences of the Charophyceae species are not yet released, mRNA sequences are available in public databases. We search the Charales sequences for ABA-related genes and identified some that encode SnRK2-like kinase and Group A-like bZIP transcription factors. This preliminary result suggests that several key genes for ABA signaling already emerged before the evolution of land plants. A complete genome sequence as well as functional analysis of the conserved molecules of the Charophyceae species would deepen our understanding of how ABA signaling evolved in land plants.

Recent progress in ABA studies has highlighted a toolkit for ABA function that is absent in algae, suggesting that emergence of the toolkit in the ancestral land plants was key to using the ubiquitous molecule as a stress hormone, enabling adaptation to the terrestrial environment. Thus, the current picture of ABA function in angiosperms must have resulted from the modification and incorporation of new molecules into the toolkit established in the ancestral land plants. For example, SLAC1 is crucial for the control of stomatal apertures by ABA in seed plants in concert with calcium signaling; however, genes encoding SLAC1-like anion channels can be found in basal land plants (Table 1 and Fig. 6). Because of the lack of genomic information from *M. polymorpha*, we cannot currently draw conclusions about whether SLAC1 coevolved with stomata; however, integration

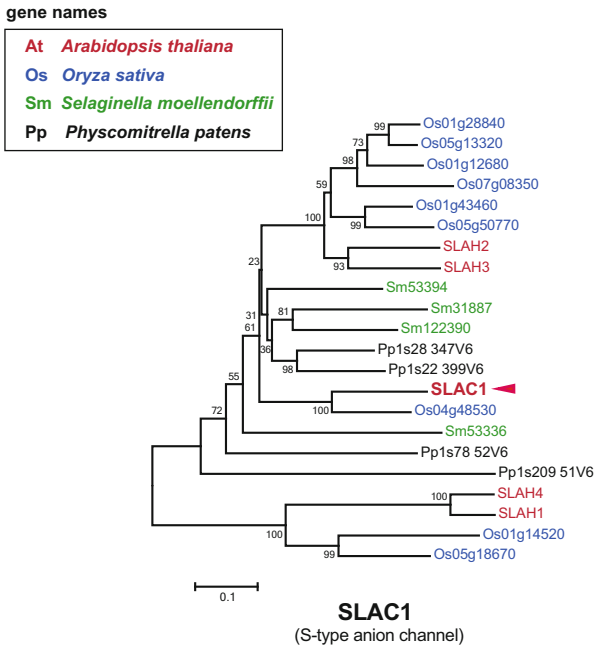

Fig. 6 Phylogenetic relationship among orthologs of SLAC1. The phylogenetic tree of SLAC1 was generated by MEGA5.0 by using the neighbor-joining method. Genes of *A. thaliana, O. sativa, S. moellendorffii*, and *P. patens* are indicated by *red*, *blue*, *green*, and *black*, respectively. The Arabidopsis SLAC1 is indicated by an *arrowhead*

of the anion channel and calcium signaling under the control of ABA-core components must be considered as a key event allowing land plants to achieve permanent hydration (homoiohydry).

The evolution of ABA signaling also enabled acquisition of seed desiccation tolerance. It appears that a common system operates vegetative (gametophyte) desiccation tolerance of bryophytes and seed (sporophyte) desiccation tolerance; however, ABA signaling in seeds has developed a novel function to operate seed dormancy and germination, which is known to involve not only ABA signaling but also GA signaling as well as light signaling. Because GA signaling evolved after ABA signaling, evolution of a cross talk between the two pathways in the reproductive organ would be an issue to address in future studies. The same is true for ABA signaling and pathogenesis. Salicylic acid (SA) plays a central role in pathogen response in angiosperms. Interaction between SA signaling and ABA signaling for pathogen responses has been suggested (Rock et al. 2010). Involvement of ABA in pathogenesis is largely unknown, although ABA and SA increase in *P. patens* upon infection by *Botrytis cinerea* (Ponce de León et al. 2012). The role of ABA in pathogen responses of basal land plants is an open question.

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Reduced Genomes from Parasitic Plant Plastids: Templates for Minimal Plastomes?

Kirsten Krause and Lars B. Scharff

Contents

Abstract Plastids are the characteristic cell organelles of plants. While movement, loss, and replacement of whole plastids have occurred in single-celled algae and some parasites derived thereof, land plants have shown more moderate twists in plastid evolution. Here, the most poignant deviations are the reduction in size and coding capacity of plastid genomes as a consequence of a heterotrophic lifestyle in haustorial parasites and mycoheterotrophic plants, which will be broadly summarized in this article as "parasitic plants". While the loss of photosynthesis genes can be easily explained with the vanishing of a photoautotrophic lifestyle, other gene losses are more difficult to reconcile with persisting regulatory and metabolic functions of the

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reduced plastids. An assessment of plastid gene essentiality using tobacco plastome mutants revealed that the catalog of losses even includes genes for the gene expression apparatus that are essential for cell viability under heterotrophic conditions. We will discuss whether these genes really are dispensable and to what degree minimal parasitic plant plastomes could be blueprints for artificial plastid genomes.

Keywords Endosymbiosis • Evolution • Parasitic plants • Plastids • Transcription • Translation

1 Introduction

The arguably most characteristic feature of plants and algae is that they gain their energy through photoautotrophic carbon fixation. The plastid compartment, where this takes place, did not evolve de novo, but has arisen through the uptake and subsequent enslavement of a cyanobacterium by a probably bi-flagellated heterotrophic ancestral eukaryote. This process, known as endosymbiosis, is nowadays as undisputed as the notion that originally a single endosymbiotic event has given rise to the three primary plastid containing lineages together known as Archaeplastida (Keeling 2010; Lane and Archibald 2008; Reyes-Prieto and Bhattacharya 2007). From these lineages, plastids have spread laterally by secondary or tertiary endosymbiosis to form further lineages of green (Euglenoids and Chlorarachniophytes) and red (Haptophytes, Cryptophytes, Stramenopiles, Dinoflagellates, and Apicomplexa) algal ancestry (Cavalier-Smith 1999, 2002; Keeling 2010; Lane and Archibald 2008). In several algal groups, plastids (and along with them photosynthesis) appear to have been lost secondarily. We owe this insight to a few species that have subsequently regained a functional plastid and with it photosynthetic activity (Janouskovec et al. 2010; Keeling 2010; Lane and Archibald 2008).

Unicellular algae with no photosynthetic activity but with a remnant cryptic plastid (Krause 2008) have also been described among the green algae (Borza et al. 2005; de Koning and Keeling 2004, 2006; Tartar and Boucias 2004), the Dinoflagellates (Matsuzaki et al. 2008; Sanchez-Puerta et al. 2007), and for *Astasia longa*, the heterotrophic relative of *Euglena gracilis* (Gockel and Hachtel 2000). Furthermore, the members of the apicomplexan group of unicellular parasites contain a cryptic plastid compartment (Lim and McFadden 2010).

In land plants, autotrophy has likewise been lost probably tens of times independently (Barkman et al. 2007; Bidartondo 2005; Merckx and Freudenstein 2010). The species that have lost the capability of photosynthetic energy production can be placed into two groups: those that obtain organic nutrients through mycoheterotrophy (i.e., the fungus-mediated degradation of organic material or uptake of nutrients from other plants via mycorrhizal fungi) (Bundrett 2009; Leake 1994) and those that have resorted to parasitizing on other land plants. The latter group absorbs their hosts' water, minerals,

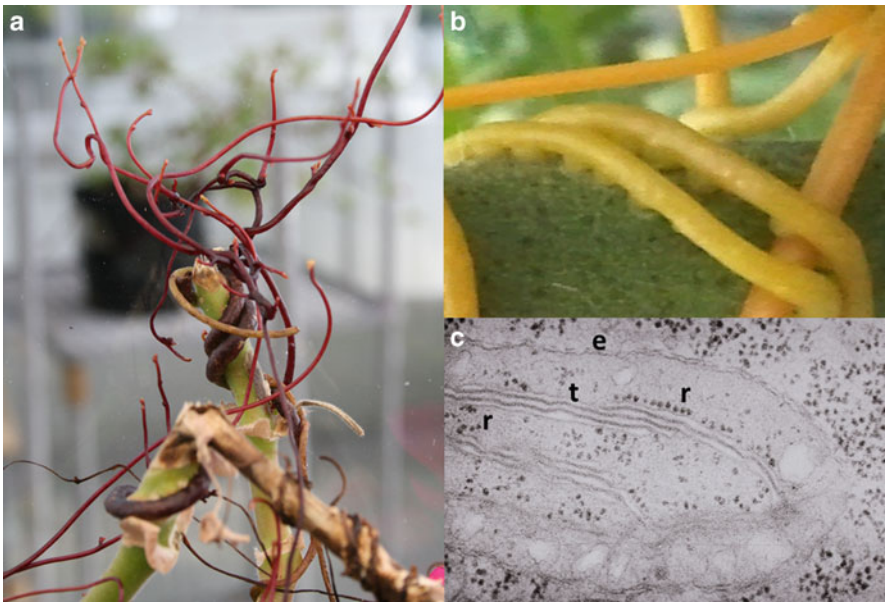

Fig. 1 Parasitic land plants. (**a**) *Cuscuta japonica* (*purple*) on *Pelargonium zonale*. (**b**) Attachment region of *Cuscuta platyloba* (*yellow*) on *Solanum lycopersicum*. The appressoria with which the parasite attaches itself to the host are visible as cone-like structures. (**c**) Electron micrograph of a *Cuscuta* plastid. *e* envelope, *t* thylakoids, *r* plastid ribosomes

and organic nutrients and thus causes substantial losses in the worldwide agricultural production (Dawson et al. 1994).

A good deal of our current knowledge on the transition to a parasitic lifestyle in land plants comes from extensive studies of the parasitic plant genus *Cuscuta*. Parasitism in this group of shoot parasites is typically, but not always, associated with a decrease or loss of photosynthetic ability (Hibberd et al. 1998; van der Kooij et al. 2000) and a concomitant reduction of leaves to minute scales (Fig. 1a, b). The roots, on the other hand, were functionally replaced by specialized feeding structures, called haustoria, which penetrate the shoots and leaves of susceptible plant hosts. Plastids of *Cuscuta* were found to exhibit a simplified ultrastructure with less or no thylakoid membranes (Hibberd et al. 1998; van der Kooij et al. 2000) (Fig. 1c), which is in line with their independence on photosynthetic electron transport.

So far, complete sequences of plastid genomes of ten parasitic or mycoheterotrophic Viridiplantae were published (see Table 1). These include four species of the dicot genus *Cuscuta* (commonly known as "dodder"), the dicot parasite *Epifagus virginiana* ("beechdrops"), the Australian underground orchid *Rhizanthella gardneri*, the bird's nest orchid *Neottia nidus-avis*, and the coralroot orchid *Corallorhiza striata*, all three representatives of the monocot angiosperms, the fern *Aneura mirabilis*, and the parasitic chlorophyte *Helicosporidium* sp. All ten plastid genomes show different degrees of reduction that are reflected by considerable differences in the plastid chromosome sizes (Table 1). Fluctuations do even occur within the closely related

Table 1 Completely sequenced plastid genomes of Viridiplantae

Species	Genbank accession number	Size (nt)	Reference
Aneura mirabilis	NC_010359	108,007	Wickett et al. (2008)
Corallorhiza striata	JX087681	137,505	Barrett and Davis (2012)
Cuscuta exaltata	NC_009963	125,375	McNeal et al. (2007)
Cuscuta gronovii	NC_009765	86,744	Funk et al. (2007)
Cuscuta obtusiflora	NC_009949	85,286	McNeal et al. (2007)
Cuscuta reflexa	NC_009766	121,521	Funk et al. (2007)
Epifagus virginiana	NC_001568	70,028	Wolfe et al. (1992)
Helicosporidium sp.	NC_008100	37,454	de Koning and Keeling (2006)
Neottia nidus-avis	NC_016471	92,060	Logacheva et al. (2011)
Rhizanthella gardneri	NC_014874	59,190	Delannoy et al. (2011)

species of the genus *Cuscuta* [Table 1 as well as Berg et al. (2003); Revill et al. (2005)]. Despite these variations, not one case is so far known among contemporary land plants, where the entire plastid genome was lost. Although the status of the plastid genome in Balanophoraceae and Rafflesiaceae has been described as ambiguous (Nickrent et al. 1997), there is so far no proof that the plastid DNA is actually missing in any members of these families. This notion has sparked the assumption that the plastome must have at least one function that is unrelated to its own maintenance and expression and that explains its retention (Howe and Purton 2007). Chloroplasts harbor several biosynthetic pathways that provide unique and important metabolites for the cell (Neuhaus and Emes 2000). Accordingly, among the functions that have been in the limelight over the years were lipid biosynthesis, heme biosynthesis, and plastid protein import. Nevertheless, the "raison d'être" for the prevalence of reduced plastid genomes has so far remained subject to speculation and the reader is referred to several reviews for more detailed information on these aspects (Barbrook et al. 2006; Howe and Purton 2007; Krause 2008, 2012; Lim and McFadden 2010).

Recent advances in cataloging essential and nonessential plastid genes by targeted plastid genome modifications in tobacco have brought up the question of whether some of the lost genes were perhaps transferred to the nucleus, in which case the gene products might still be active in the plastids. We will in the following focus on the two essential processes of transcription and translation and discuss the likelihood of a transfer to the nuclear genome.

2 Transcription in Higher Plant Plastids

Most genes on the plastid genome are transcribed in units called operons. Each operon is preceded by one or more promoters that are responsible for attracting a DNA-directed RNA polymerase and enabling faithful transcription initiation. There are numerous nucleus-encoded transcription factors that bind to the promoters and thus facilitate the binding of the RNA polymerase core enzyme. The best studied

Table 2 Plastid-encoded components of the transcription complex and their presence or absence in parasitic plants

Gene name	Essential for photoautotrophic growth?	Lost in
rpoA	Yes (Krause et al. 2000)	*C.g.*, *C.o.*, *C.s.*, *E.v.*, *N.n.*, *R.g.*
rpoB	Yes (Krause et al. 2000)	*C.g.*, *C.o.*, *C.s.*, *E.v.*, *N.n.*, *R.g.*
rpoC1	Yes (Krause et al. 2000)	*C.g.*, *C.o.*, *C.s.*, *E.v.*, *N.n.*, *R.g.*
rpoC2	n.d.	*C.g.*, *C.o.*, *C.s.*, *E.v.*, *N.n.*, *R.g.*

n.d., not determined; *C.g.*, *Cuscuta gronovii*; *C.o.*, *Cuscuta obtusiflora*; *C.s.*, *Corallorhiza striata*; *E.v.*, *Epifagus virginiana*; *N.n.*, *Neottia nidus-avis*; *R.g.*, *Rhizanthella gardneri*. Essentiality of the genes is defined here as essential for chloroplast biogenesis, i.e., necessity under autotrophic growth conditions

transcription factors are those that originate from the cyanobacterial ancestors and that are designated "sigma-like factors" (Allison 2000). The genome of *Arabidopsis thaliana* contains six genes for sigma-like factors, AtSIG1-6, whose individual functions have been elucidated quite well (e.g., Favory et al. 2005; Ishizaki et al. 2005; Loschelder et al. 2006; Privat et al. 2003; Schweer et al. 2010; Yao et al. 2003; Zghidi et al. 2007). In addition to these six proteins, transcription factors of eukaryotic origin have more recently been discovered in the plastids (Schwacke et al. 2007; Wagner and Pfannschmidt 2006), but their function and binding characteristics are still largely unknown.

In contrast to this variety of promoter binding proteins, the actual transcription process in plastids is executed by three polymerases at most: one plastid-encoded enzyme, the PEP, and one or two nucleus-encoded polymerases, the NEPs (Cahoon and Stern 2001; Liere et al. 2011). The PEP is a multi-subunit enzyme and is encoded by four plastid genes: *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* (Table 2). These genes are homologous to the eubacterial multi-subunit RNA polymerase and transcribe genes that bear similarity to prokaryotic promoters (PEP promoters) (Hess and Börner 1999). In monocot leaves, which display a gradient with respect to plastid developmental stages, the activity of the PEP is highest in leaf segments with mature chloroplasts, fitting the notion that it predominantly, but not exclusively, transcribes photosynthesis-related genes (Mullet 1993). In Arabidopsis, PEP was recently also shown to selectively transcribe the *rrn* operon during seedling germination (Demarsy et al. 2012).

Besides the "prokaryotic" subunits of PEP, ten additional nucleus-encoded proteins were recently found to be tightly associated with this polymerase (Steiner et al. 2011). These proteins, dubbed "PAPs" (which stands for *P*EP-*a*ssociated *p*roteins), were all found to be essential for the development of photosynthetically active mature chloroplasts (see references in Table 3). While the PAPs together with the plastid-encoded *rpo*-gene products are thought to constitute the basic PEP complex (Steiner et al. 2011), sigma-like factors and other proteins are likely to be associated either transiently or more peripherally. Some of these proteins were consistently detected in a transcriptionally active fraction of mature chloroplasts (Krause and Krupinska 2000). This fraction that was termed TAC (Hallick et al. 1976; Igloi and Kössel 1992) or later PTAC (Pfalz et al. 2006) (*p*lastid *t*ranscriptionally *a*ctive *c*hromosome) retains endogenous transcription activity during the isolation process (Krause and Krupinska 2000). In addition to fulfilling transcription-related tasks, "PTAC-proteins" appear to provide

Table 3 Nuclear-encoded components of the plastid transcription complex and their presence or absence in two *Cuscuta* species based chiefly on transcriptome analysis (Krause, unpublished data)

Gene name (protein function)	AGI code	Essential for photoautotrophic growth?	Found in	
			C. reflexa	*C. gronovii*
rpoTmp (dual-targeted (p+m) NEP)	At5g15700	No (Hricova et al. 2006)	+	+
rpoTp (plastid-targeted NEP)	At2g24120	Yes (Baba et al. 2004)	+	–
AtSIG1 (σ-subunit of PEP)	At1g64860	No (Privat et al. 2003)	+	–
AtSIG2 (σ-subunit of PEP)	At1g08540	Yes (Privat et al. 2003)	+	–
AtSIG5 (σ-subunit of PEP)	At5g24120	Yes (Yao et al. 2003)	+	–
AtSIG6 (σ-subunit of PEP)	At2g36990	No (Ishizaki et al. 2005)	+	–
AtSIG3 (σ-subunit of PEP)	At3g53920	No (Privat et al. 2003)	–	–
AtSIG4 (σ-subunit of PEP)	At5g13730	No (Favory et al. 2005)	–	–
PTAC3 (PEP-associated protein (PAP)1)	At3g04260	Yes (Myouga et al. 2010)	+	–
PTAC10 (PAP3)	At3g48500	Yes (Steiner et al. 2011)	+	–
FSD3 (PAP4)	At5g23310	Yes (Myouga et al. 2010)	+	–
PTAC12 (PAP5)	At2g34640	Yes (Pfalz et al. 2006)	+	–
FLN1 (PAP6)	At3g54090	Yes (Steiner et al. 2011)	+	–
PTAC14 (PAP7)	At4g20130	Yes (Steiner et al. 2011)	+	–
PTAC6 (PAP8)	At1g21600	Yes (Pfalz et al. 2006)	+	–
FSD2 (PAP9)	At5g51100	Yes (Myouga et al. 2010)	+	–
TRXZ (PAP10)	At3g06730	Yes (Arsova et al. 2010)	+	–
PTAC2 (PAP2)	At1g74850	Yes (Pfalz et al. 2006)	–	–

p plastid, *m* mitochondrial, *SIG* sigma-like factor, *PAP* PEP-associated protein, *PTAC* plastid transcriptionally active chromosome component, *FSD* iron superoxide dismutase, *FLN* pfkB-2 fructokinase, *TRX* thioredoxin. Essentiality of the genes is defined here as essential for chloroplast biogenesis, i.e., necessity under autotrophic growth conditions

functions connected to DNA condensation and architecture (Melonek et al. 2012) and the anchoring of the plastid DNA molecules to the thylakoid membranes (Ingelsson and Vener 2012; Majeran et al. 2012). Also, it was discussed whether they may be necessary to adapt chloroplast transcription to changing redox balances (Schroter et al. 2010).

The plastid *rpoBC* operon and the separately encoded *rpoA* gene are transcribed by a NEP. The two different NEP enzymes that are active in the plastids consist of only a single subunit each and resemble T7-phage type RNA polymerases (Liere et al. 2011). These proteins are encoded by the two nuclear genes *rpoTp* and *rpoTmp* (see Table 3). While earlier results from tobacco indicated that the NEPs transcribe a set of genes that only to a small part overlap with that of the PEP (Hajdukiewicz et al. 1997; Krause et al. 2000; Legen et al. 2002), a recent analysis of the PEP-lacking plastids of the *albostrians* mutant of barley showed that most genes (including photosynthetic genes) have both PEP and NEP promoters (Zhelyazkova et al. 2012). This means that there could be considerable functional

redundancy among the plastid RNA polymerases and that division of labor could mainly secure a higher efficiency of gene expression in situations of intense metabolic activity.

2.1 *Knockout of Genes Coding for Transcriptional Apparatus Components in Photosynthetic Angiosperms*

Homoplastomic knockout mutants for the plastid-encoded *rpo*-genes were generated in tobacco already more than a decade ago (DeSantis-Maciossek et al. 1999; Hajdukiewicz et al. 1997). Phenotypically, these deletions lead to a loss of photosynthetic capacity that is accompanied by an off-white phenotype (DeSantis-Maciossek et al. 1999) and—at the molecular level—by a reduction in the amount of transcripts of photosynthesis genes (Krause et al. 2000; Legen et al. 2002). Similar phenotypes were seen when the nucleus-encoded PAPs were deleted [summarized in Table 3 and in the recent work by Steiner et al. (2011)], suggesting that they are essential for the transition of proplastids into mature chloroplasts. In contrast, only two of the six SIG genes caused severe phenotypes when knocked out (Table 3).

2.2 *Loss of Genes Coding for Transcriptional Apparatus Components in Parasitic Plants*

The selective pressure that rests on photosynthesis is seen as one of the driving forces for a highly conserved plastid genome in photosynthetic land plants (Bock 2007). With the relaxation of this pressure, the plastid gene expression apparatus of parasitic plants was prone to be relieved from the need for a highly efficient and tightly regulated expression of photosynthesis genes. The finding that the transcription patterns in plastids of three *Cuscuta* species resemble each other remarkably despite significant differences in plastid coding capacity (Berg et al. 2003) provides one line of support for this premiss. The loss of the plastid *rpo* genes encoding the PEP core in many parasitic plants and algae along with the PEP-dependent photosynthesis genes (reviewed by Krause (2011); see also Table 2) can be seen as another logical consequence of this reduced selective pressure. In contrast, the losses of all four *rpo* genes in the two *Cuscuta* species *C. gronovii* and *C. obtusiflora* that have retained most photosynthesis genes are a unique case, since nowhere else PEP subunit losses concurred with a retention of functional and transcribed PEP-dependent genes. The explanation for this was found in a switch from PEP-based to NEP-based transcription that allowed photosynthesis genes to be transcribed in the absence of PEP (Berg et al. 2004; Funk et al. 2007). Indications that transcripts for at least one of the two plastid NEPs, the one encoded by the *rpoTmp* gene, are present in *C. gronovii* (Table 3), support this conclusion. Interestingly, the protein product of *rpoTmp* is dually targeted to both plastids and mitochondria, while the protein encoded by *rpoTp*, for which no trace of its existence in *C. gronovii* was found so far, is imported only into the plastids. Like with the PEP, this may indicate that the *rpoTp* target genes in the plastids were

lost or have changed their promoters. If this were true, an analysis of all NEP genes and their promoters in *Cuscuta* could facilitate the assignment of specific target genes to the two NEPs in photosynthetic higher plants. It may also be noteworthy that neither transcripts for the six sigma-like proteins (SIG1-6) nor any of the ten other PEP-associated proteins (PAP1-10) were found in *C. gronovii* (Table 3). However, without direct investigations of the nuclear genome sequence of this species, it will remain unresolved whether the corresponding genes are, in fact, missing or whether these genes are present but not expressed.

In contrast to *C. gronovii*, *C. reflexa* expresses the genes for at least four of the six sigma-like transcription factors from the nuclear genome (Table 3). The two proteins for which no transcripts could be found were SIG3 and SIG4. While SIG3 transcribes specifically the *psbN* gene (Zghidi et al. 2007), SIG4 was reported to be responsible for *ndhF* gene transcription (Favory et al. 2005). The latter gene is lacking from the plastid genome of *C. reflexa*, making SIG4 likewise obsolete. In addition to the sigma-like transcripts, evidence for the expression of all but one of the PAPs was found in *C. reflexa* (Table 3). That their retention coincides with the retention of the PEP in this species corroborates the conclusions made by Steiner et al. (2011) that these proteins (maybe with the exception of PAP2) are necessary for PEP transcription.

3 The Plastid Translational Apparatus of Higher Plants

The translational apparatus of plastids is derived from their bacterial ancestors. It consists of bacterial-type 70S ribosomes, tRNAs, and translation factors, whereas the regulation is thought to be executed by eukaryotic protein families, e.g., pentatricopeptide repeat (PPR) proteins (Barkan 2011; Peled-Zehavi and Danon 2007). All rRNAs, tRNAs, and (typically) 21 of 57 ribosomal proteins are encoded in the plastid genome (Bock 2007). The remaining components of the plastid translational apparatus are encoded in the nucleus. Plastid proteins' synthesis in most plants is strictly essential (Ahlert et al. 2003; Rogalski et al. 2006), which in this context means that it is indispensable for cell division under autotrophic as well as under heterotrophic conditions. Known exceptions are some Brassicaceae species (Zubko and Day 1998) and the Poaceae family (Han et al. 1992; Hess et al. 1994). However, this does not imply that all ribosomal components are needed to the same degree. From reverse genetic studies in the model plant *Nicotiana tabacum*, for example, it is known that the knockouts of some ribosomal protein genes (Fleischmann et al. 2011; Rogalski et al. 2008b) and tRNA genes (Rogalski et al. 2008a) only impair growth and photosynthesis, but are not essential for plastid protein synthesis.

Parasites have lost up to six of the standard set of 21 genes for ribosomal proteins [*E. virginiana*, Wolfe et al. (1992)] and up to 21 out of 30 plastid genes for tRNAs [*R. gardneri*, Delannoy et al. (2011)]. Unlike photosynthetic plants, where high translational capacity is needed during the transition from young to mature chloroplasts for the buildup of the photosynthetic complexes, parasitic plants

might actually be able to tolerate the loss of some genes for ribosomal proteins and tRNAs. However, this is still heavily debated. *N. tabacum* can grow heterotrophically on sucrose-containing medium (Fleischmann et al. 2011), which mimics the heterotrophic lifestyle of the parasitic species. A comparison of the minimal gene set in this model plant and the strongly reduced gene sets in the parasites could indicate (a) which genes may have been lost due to their being nonessential under heterotrophic growth conditions or (b) which ones were likely transferred to the nuclear genome and their products imported back into the plastids or were replaced by nuclear-encoded genes of different origin, respectively.

3.1 Loss of Ribosomal Proteins from Plastids of Parasitic Plant Species

In *N. tabacum* three ribosomal protein genes, *rpl33*, *rpl36*, and *rps15*, are not essential for chloroplast biogenesis (Fleischmann et al. 2011; Rogalski et al. 2008b). The same genes are also dispensable in *Escherichia coli* (Bubunenko et al. 2006; Maeder and Draper 2005; Maguire and Wild 1997) demonstrating the high conservation of the 70S ribosomes in bacteria and plastids. Two of these nonessential genes are missing in the plastid genomes of several parasitic species: *rpl33* in *Helicosporidium* sp. and *R. gardneri* and *rps15* in *E. virginiana*, *Helicosporidium* sp., and *R. gardneri* [see Table 4 and Krause (2011)]. The knockout of these two genes impaired plastid translation under ambient growth conditions only weakly (Fleischmann et al. 2011; Rogalski et al. 2008b), which indicates that ribosomes of the parasitic species could function without them. The knockout of *rpl36*, on the other hand, massively impaired translation in *N. tabacum* (Fleischmann et al. 2011), which may explain why this gene is retained in all of the parasitic plant species.

Besides *rpl33* and *rps15*, parasitic species have lost seven other genes (Table 4). Six of them were demonstrated to be essential in *N. tabacum* (Fleischmann et al. 2011; Rogalski et al. 2006, 2008b). The seventh gene, *rpl14*, which was lost from the plastid genome in *E. virginiana*, is probably also essential for translation, judging from its essentiality in *E. coli* (Baba et al. 2006). The questions arising now are whether these genes have become dispensable and, if so, whether this has affected plastid ribosome assembly and translation efficiency. The lack of a sequenced nuclear genome of a parasitic plant makes it impossible to conclusively answer whether these genes could have been transferred to the nucleus. In photosynthetic plants, such transfers of plastid DNA fragments to the nucleus happen at ample frequency (Leister and Kleine 2011), and examples where transferred genes have acquired a plastid target peptide that directs the encoded protein back to the organelle do exist. For example, in *Pisum sativum* and *Populus alba*, respectively, the two genes *rpl22* and *rpl23* were functionally transferred to the nuclear genome (Gantt et al. 1991; Ueda et al. 2007). Other "missing" genes were replaced by nuclear-encoded copies of different origin. The *rpl23* gene coding for the ribosomal protein L23, for instance, was functionally replaced in *Spinacia oleracea* by an

Table 4 Plastid-encoded components of the translation complex and their presence or absence in parasitic plants

Gene name	Essential for cell viability in *N. tabacum*?	Lost in
rpl2	n.d.	–
rpl14	n.d.	*E.v.*
rpl16	n.d.	–
rpl20	Yes (Rogalski et al. 2008b)	–
rpl22	Yes (Fleischmann et al. 2011)	*E.v.*, *H.*sp., *R.g.*
rpl23	Yes (Fleischmann et al. 2011)	*C.e.*, *C.g.*, *C.o.*, *C.r.*, *E.v.*, *H.*sp.
rpl32	Yes (Fleischmann et al. 2011)	*C.g.*, *C.o.*, *E.v.*, *R.g.*
rpl33	No (Rogalski et al. 2008b)	*H.*sp., *R.g.*
rpl36	No (Fleischmann et al. 2011)	–
rps2	Yes (Rogalski et al. 2008b)	*H.*sp.
rps3	Yes (Fleischmann et al. 2011)	–
rps4	Yes (Rogalski et al. 2008b)	–
rps7	n.d.	–
rps8	n.d.	–
rps11	n.d.	–
rps12	n.d.	–
rps14	Yes (Ahlert et al. 2003)	–
rps15	No (Fleischmann et al. 2011)	*E.v*, *H.*sp., *R.g.*
rps16	Yes (Fleischmann et al. 2011)	*A.m.*, *C.e.*, *C.g.*, *C.o.*, *C.r.*, *E.v.*, *H.*sp., *N.n.?*, *R.g.*
rps18	Yes (Rogalski et al. 2006)	*H.*sp., *N.n.?*
rps19	n.d.	–

The plastid-encoded set of ribosomal protein genes was determined for all nine parasitic species of the Viridiplantae listed in Table 1. n.d., not determined; ?, pseudogene suspected; *A.m.*, *Aneura mirabilis*; *C.e.*, *Cuscuta exaltata*; *C.g.*, *Cuscuta gronovii*; *C.o.*, *Cuscuta obtusiflora*; *C.r.*, *Cuscuta reflexa*; *C.s.*, *Corallorhiza striata*; *E.v.*, *Epifagus virginiana*; *H.*sp., *Helicosporidium* sp.; *N.n.*, *Neottia nidus-avis*; *R.g.*, *Rhizanthella gardneri*. Essentiality of the genes in *N. tabacum* is defined as essential for cell division, i.e., essential under autotrophic and heterotrophic conditions

additional copy of a gene coding for the cytosolic L23, whose product is targeted to the plastids (Bubunenko et al. 1994). The *rps16* gene in *P. alba* and *Medicago truncatula* was replaced by a nuclear gene, which encodes a S16 protein that is dually targeted to mitochondria and plastids (Ueda et al. 2008). The same authors also demonstrated that a nuclear-encoded dual-targeted S16 protein exists in *Arabidopsis thaliana*, *Solanum lycopersicum*, and *Oryza sativa*, which still have a plastid-encoded S16 protein.

So far, however, all attempts at finding evidence for any of the missing ribosome protein genes in the nucleus of parasitic plant species or their transcriptome by us and others (Delannoy et al. 2011) have to our knowledge been unsuccessful. Therefore, alternative explanations have to be taken into consideration. The acquisition of a dual targeting signal peptide by the originally mitochondrial copies, as exemplified by the *rps16* gene (see above), could have enabled parasitic plants to functionally replace missing ribosomal proteins or explain why the corresponding plastid genes

became futile in the first place. Theoretically, however, not only gene transfer or replacement by genes for ribosomal proteins from a different origin is possible, but also compensatory mutations in the rRNA and newly developed genes to preserve the functionality of the plastid ribosomes. The plastid rRNAs are highly conserved also in parasitic species, but do exhibit differences. At least some of the plastid-specific ribosomal proteins are known to compensate for differences in the rRNA (Sharma et al. 2007; Tiller et al. 2012). It is possible that new proteins evolved in parasitic plants, which could compensate not only for changes in the rRNA, but also for the lack of the ribosomal proteins of the standard set for 70S ribosomes.

3.2 tRNAs in Parasitic Plant Plastids

Plastids possess a reduced tRNA set. Only 30 tRNAs decode 61 codons (Bock 2007), which is less than the minimum set required according to the wobble rules (Crick 1966). Reverse genetic studies in *N. tabacum* demonstrated that the minimum tRNA set for the standard genetic code consists of 25 tRNAs (Alkatib et al. 2012a), i.e., it could be reduced further compared to the already reduced set in typical plant plastids. This reduction is possible using the following strategies: (1) for all family codon boxes, i.e., four codons sharing the same first nucleotides and coding for the same amino acid, only one tRNA with a U at the wobble position is sufficient if the superwobble mechanism is used (Rogalski et al. 2008a). The exception is $tRNA^{Arg}$(ACG), which has an inosine (modified A) at the wobble position, but can also read all four codons in one family box. (2) All mixed codon boxes, i.e., codon boxes coding for different amino acids, are decoded by tRNAs capable of reading two codons by using the wobbling mechanism, i.e., tRNAs with U or G at the wobbling position. Using this strategy, one tRNA is sufficient to decode any amino acids that are represented by one to four codons. For arginine, leucine, and serine, which are each encoded by six codons, two tRNAs are sufficient. Only for the AUN box coding for isoleucine and methionine, more tRNAs are necessary: the $tRNA^{fMet}$(CAU) as initiator tRNA reading AUG start codons, $tRNA^{Met}$(CAU) for internal AUG methionine codons, $tRNA^{Ile}$(CAU) for AUA isoleucine codons, and $tRNA^{Ile}$(GAU) for AUC and AUU isoleucine codons (Alkatib et al. 2012b). $tRNA^{Ile}$(UAU) would be able to read all four codons by using the superwobble mechanism, but would be unable to distinguish between methionine and isoleucine codons. Therefore, $tRNA^{Ile}$(CAU) is used, which has the C at the wobble position modified to lysidine, which restricts its binding capacity to the AUA isoleucine codon. Of the four tRNAs decoding the AUN box, only $tRNA^{Ile}$(GAU) is able use the wobble mechanism and therefore able to read two codons.

Astonishingly, only one of the sequenced parasitic species of the Viridiplantae is using this strategy to restrict the tRNA set to the necessary minimum: the parasitic, non-photosynthetic green alga *Helicosporidium* sp. (de Koning and Keeling 2006) (see Table 5). This species lacks only the genes for the five tRNAs, which are dispensable by maximum use of the wobble and superwobble mechanisms:

Table 5 Plastid-encoded tRNAs and their presence or absence in parasitic plants

Gene name	Essential for cell viability in *N. tabacum*?	Lost in
trnA-UGC	Yes (Alkatib et al. 2012b)	*E.v.*, *C.g.*, *C.o.*, *R.g.*
trnC-GCA	Yes (Legen et al. 2007)	*E.v.*
trnD-GUC	n.d.	–
trnE-UUC	n.d.	–
trnF-GAA	n.d.	–
trnG-GCC	No (Rogalski et al. 2008a)	*E.v.*, *H.*sp., *R.g.*
trnG-UCC	Yes (Rogalski et al. 2008a)	*C.g.*, *C.o.*, *E.v.*, *R.g.*
trnH-GUG	n.d.	*R.g.*
trnI-CAU	Yes (Alkatib et al. 2012a)	–
trnI-GAU	Yes (Alkatib et al. 2012a)	*C.g.*, *C.o.*, *E.v.*, *N.n.*, *R.g.*
trnK-UUU	n.d.	*C.e.*, *C.g.*, *C.o.*, *C.r.*, *E.v.*, *R.g.*
trnL-CAA	No (Alkatib et al. 2012b)	*H.*sp., *R.g.*
trnL-UAA	Yes (Alkatib et al. 2012b)	*E.v.*, *R.g.*
trnL-UAG	Yes (Alkatib et al. 2012b)	*R.g.*
trnfM-CAU	Yes (Alkatib et al. 2012a)	–
trnM-CAU	Yes (Alkatib et al. 2012a)	*R.g.*
trnN-GUU	Yes (Legen et al. 2007)	*R.g.*
trnP-UGG	n.d.	*N.n.*, *R.g.*
trnQ-UUG	n.d.	–
trnR-ACG	Yes (Alkatib et al. 2012b)	*C.g.*, *C.o.*, *R.g.*
trnR-UCU	Yes (Alkatib et al. 2012b)	*E.v.*, *R.g.*
trnS-GCU	Yes (Alkatib et al. 2012b)	*R.g.*
trnS-GGA	No (Alkatib et al. 2012b)	*E.v.*, *H.*sp., *R.g.*
trnS-UGA	Yes (Alkatib et al. 2012b)	*R.g.*
trnT-GGU	No (Alkatib et al. 2012b)	*C.s.*, *E.v.*, *H.*sp., *R.g.*
trnT-UGU	Yes (Alkatib et al. 2012b)	*E.v.*, *R.g.*
trnV-GAC	No (Alkatib et al. 2012b; Corneille et al. 2001)	*E.v.*, *H.*sp., *R.g.*
trnV-UAC	Yes (Alkatib et al. 2012b)	*C.g.*, *C.o.*, *E.v.*, *N.n.*, *R.g.*
trnW-CCA	n.d.	–
trnY-GUA	n.d.	–

The plastid tRNA gene content was determined for all nine parasitic species of the Viridiplantae listed in Table 1. n.d., not determined; ?, pseudogene; *A.m.*, *Aneura mirabilis*; *C.e.*, *Cuscuta exaltata*; *C.g.*, *Cuscuta gronovii*; *C.o.*, *Cuscuta obtusiflora*; *C.r.*, *Cuscuta reflexa*; *C.s.*, *Corallorhiza striata*; *E.v.*, *Epifagus virginiana*; *H.*sp., *Helicosporidium* sp.; *N.n.*, *Neottia nidus-avis*; *R.g.*, *Rhizanthella gardneri*. Essentiality of the genes in *N. tabacum* is defined as essential for cell division, i.e., essential under autotrophic and heterotrophic conditions

trnG-GCC, *trnL-CAA*, *trnS-GGA*, *trnT-GGU*, and *trnV-GAC*. In contrast, the plastid genome of the parasitic orchid *Corallorhiza striata*, a species which only recently evolved a heterotrophic lifestyle, has only a moderate reduction in size compared to autotrophic relatives. It has lost only one nonessential tRNA gene, *trnT-GGU* (Barrett and Davis 2012).

The parasitic liverwort *Aneura mirabilis* (Wickett et al. 2008) features the complete tRNA set of a typical plant. It even has an additional $tRNA^{Arg}$(CCG) that is also present in its autotrophic relative *Marchantia polymorpha* but not in

most higher plants. In the other seven species, for which sequenced plastid genomes are available, tRNA genes were lost from the plastid genome, which are essential for protein synthesis (Table 5). There are even curious cases where the U containing tRNA capable of superwobbling was lost, whereas the G containing tRNA, which is nonessential in *N. tabacum*, was preserved, e.g., $tRNA^{Gly}$ and $tRNA^{Val}$ in *C. gronovii* and *C. obtusiflora*. In these examples two of four codons for glycine and valine, respectively, cannot be read by the plastid-encoded tRNA.

R. gardneri possesses the most strongly reduced tRNA set: its plastid genome codes for only nine tRNAs. These are sufficient to decode just nine of 20 amino acids. For two of them, isoleucine and methionine, not even all codons can be read. Consequently, in both this exceptional orchid that lives exclusively underground and in the other six parasitic land plant species not even an altered genetic code could explain the reduced tRNA set, because they lack tRNAs for some of the amino acids. Accordingly, the codon usage and genetic code were assessed as being non-adapted to the reduced tRNA set in these parasitic plants (Delannoy et al. 2011; Funk et al. 2007; Wolfe et al. 1992).

It has been suggested that *trnE* may be the only gene that is found in all plastid genomes, regardless of the degree of plastid genome reductions (Barbrook et al. 2006), and so far, all sequenced plastomes fulfill this prediction. However, answer for the question, whether this indeed—as discussed by Barbrook and colleagues—is based on the fact that *trnE* is the only plastid tRNA that fulfills a second role, namely, in the heme biogenesis pathway (a hypothesis that has been, meanwhile, contested by the existence of a *trnE*-independent heme biosynthesis pathway in Apicomplexa) or whether other reasons prevail or even whether *trnE*-less plastids are possible, will hopefully be revealed as the set of cryptic plastid genome sequences increases.

3.3 tRNA Import

The lack of essential plastid-encoded tRNAs, especially in cases, in which no tRNAs for an amino acid is encoded in the plastid genome at all, makes tRNA import into plastids in these species almost imperative. tRNA import is widespread in mitochondria (Duchêne et al. 2009; Schneider 2011) but was never experimentally proven for plastids. Plastid genomes usually code for all necessary tRNAs and accordingly no imported tRNAs were detected experimentally in *N. tabacum* (Lung et al. 2006; Rogalski et al. 2008a). In mitochondria, on the other hand, the number of imported tRNAs varies from tRNAs for one amino acid to tRNAs for all amino acids. The imported tRNAs are typically cytosolic tRNAs. Their import into plant mitochondria depends probably on aminoacyl tRNA synthetases, receptors for protein import, and a voltage-dependent anion channel (Schneider 2011). The range of imported tRNAs varies even in related species. This indicates that either the mitochondrial tRNA import machinery can evolve quickly and did this often independently or a cryptic machinery exists in many eukaryotic lineages, which is only activated in some of them (Schneider 2011).

The experimentally verified essentiality of plastid tRNAs in *N. tabacum* (Alkatib et al. 2012a, b; Legen et al. 2007; Rogalski et al. 2008a) indicates that tRNA import cannot be activated by the simple lack of a tRNA gene. Still, the incomplete tRNA sets in parasitic species (Table 5) indicate that tRNA import into their cryptic plastids must exist. Interestingly, the parasitic green alga *Helicosporidium* sp. does not only have a functional set of tRNAs decoded in the plastid genome (de Koning and Keeling 2006), but also has a functional set of tRNA genes in its mitochondria (Pombert and Keeling 2010). This situation contrasts with that in most higher plants, where no sufficient tRNA set is encoded in the mitochondrial genome. It is possible that *Helicosporidium* sp. lacks the mitochondrial tRNA import machinery, whereas it is present in higher plants and could be adapted for plastid tRNA import in the parasitic species.

4 Conclusions and Perspectives

The reduced genomes of parasitic organisms like the human pathogen *Mycoplasma genitalium* (Gibson et al. 2008) are used to design minimal genomes. The comparison of the reduced plastid genomes of parasitic plants could in a similar way be useful to determine which genes are essential and what is the minimal gene set necessary for a functional plastid genome.

The division of transcriptional responsibility between one PEP enzyme and two NEPs seems to be necessary in photosynthetic organisms while parasitic species like *C. gronovii* seem to thrive without. This suggests that a more elaborate gene regulatory network is necessary to grant fast adaptation of the photosynthetic complexes to changing light conditions or other environmental cues, but that non-photosynthetic plastids in parasites—and perhaps also other strong sink tissues—may be able to function without these additional layers of regulation.

Whereas parasitic plastid genomes provide useful information about minimal gene sets for the transcriptional apparatus, it is currently unclear whether this is also true for the translational machinery. The amount of plastid protein synthesis in parasitic species is much lower compared to photosynthetic species. Therefore, theoretically a less efficient translation could be tolerated, but, actually, only a few gene losses from the plastid genome in the parasitic species can be explained this way. In fact, many genes essential for translation are lost. One would expect that their function is fulfilled by nuclear-encoded genes, whose products are imported into plastids. However, in the absence of information about the genome of the parasitic species, the nature of these genes remains unknown. In addition, the conservation of nonessential genes in some of the strongly reduced plastid genomes of parasitic species provokes questions about the selective pressure involved.

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Part III
Physiology

Nucleotides and Nucleosides: Transport, Metabolism, and Signaling Function of Extracellular ATP

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Contents

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Abstract The focus of this work is to summarize the current knowledge related to transport of nucleotides and nucleosides in and outside of cells, including the vacuole, and functions and metabolism of extracellular ATP (eATP). Thereby mechanisms on how nucleotides and their derivatives can leave cells and how their export is stimulated are described. Furthermore, effects exerted by eATP are listed including the corresponding downstream signaling events. The signal molecule eATP is removed from the extracellular space by the hydrolytic activity of several degrading enzymes like E-NTPDases, phosphatases, 5′ nucleotidases, and nucleoside hydrolases. The final reaction products of these enzymes, nucleosides and nucleobases, are then reimported into the cells to supply the corresponding cytosolic pools. The family of equilibrative nucleoside transporters (ENTs), responsible for nucleoside reimport into the cells, is presented in more detail. Finally, plant ENT family members residing at the plasma membrane or the tonoplast are compared with human and yeast homologs at the biochemical and physiological level.

1 Introduction

Nucleotides and nucleosides are highly valuable resources for the cell because they function as energy providers, signals, and building blocks for nucleic acids as well as the plant hormone cytokinin. The majority of cellular nucleotides are bound in the nucleic acids DNA and RNA. Whereas the cellular DNA content is invariant, RNA, especially ribosomal RNA (rRNA) levels, can be adapted to nutritional conditions such as limiting nitrogen or phosphate. Free nucleotides serve in a wealth of anabolic and catabolic reactions and are components of cofactors such as nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), s-adenosylmethionine (SAM), or coenzyme A (CoA) (Zrenner et al. 2006). Furthermore, nucleotide levels in and outside of cells act as signals. In the cell relative adenylate contents determine the cellular energy charge whereas outside ATP seems to be the main signal.

In the last few years, extracellular ATP (eATP) has been established as a signaling molecule in plants. eATP may enter the apoplast through secretory vesicles or by specialized transporters and is supposed to bind to ATP receptors, similar to the situation in animals (Burnstock, 2009). However, no such receptor homologs have been identified in plant genomes so far. Intertwined with eATP signaling is the so-called extracellular salvage (Fig. 1). The salvage entails the breakdown of AMP originating from the apyrase activity to adenosine and adenine and their transport back into the cytosol. Equilibrative nucleoside transporters (ENTs) are involved in nucleoside import into cells. Furthermore, members of this protein family are found in endomembranes of plants but also of mammals and fungi where they seem to be involved in the recycling of nucleic acids.

ATP and ADP are precursors for the synthesis of the phytohormone cytokinin and responsible for the structural similarity between cytokinins and purines. A variety of similar structures, all representing N-6 substituted aminopurines, are found in plants as active cytokinins, influencing a multitude of biological processes.

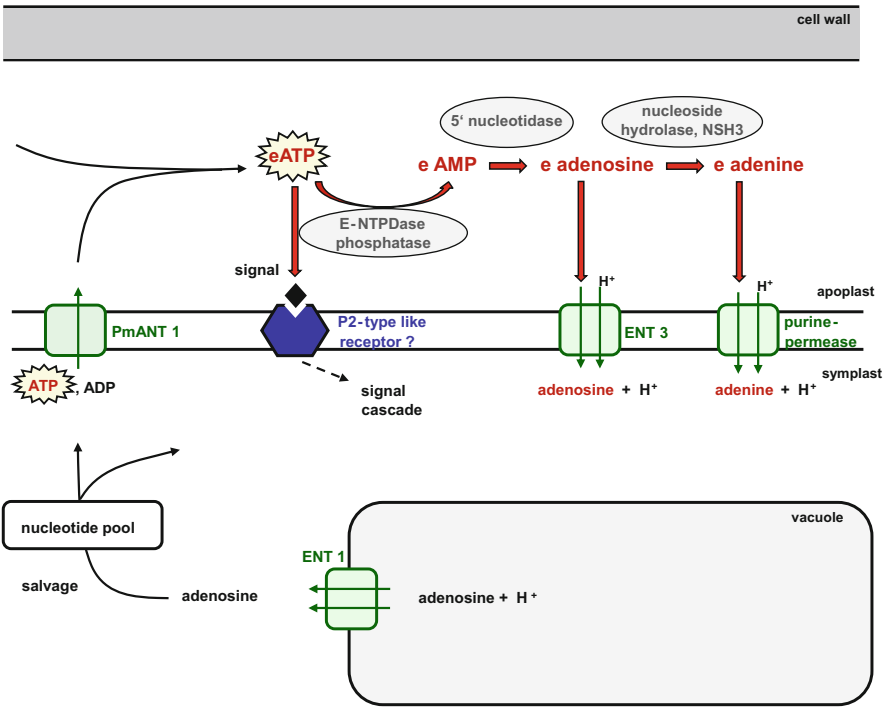

Fig. 1 Schematic overview of cellular nucleoside transport processes and connected metabolic pathways. Extracellular ATP (eATP) appears in the apoplast after export by PM-ANT1 or other mechanisms as discussed in the text. eATP may then lead to signal transduction via a so far unknown receptor or becomes degraded by the action of E-NTPDase or phosphatase. The resulting AMP is then further processed by 5′ nucleotidase to extracellular adenosine (eadenosine) and further to eadenine by the nucleosidase NSH3. Both adenosine and adenine can be reimported into cells by nucleoside transporters (ENT3) or purine permeases and then enter the salvage pathway. The vacuole may also serve as a source for nucleosides stemming from RNA degradation which are exported by ENT1 and feed in the cytosolic nucleotide pool. *PM-ANT1* plasma membrane nucleotide transporter 1, *ENT* equilibrative nucleoside transporter, *NSH3* nucleoside hydrolase 3, *eATP*/*eadenosine*/*eadenine* extracellular ATP/adenosine/adenine

Whereas synthesis and degradation of cytokinins have been studied in detail, not much is known about corresponding transport processes.

2 Export Mechanisms for Nucleotides

The simplest exit path for nucleotides is through a damaged plasma membrane. The concentration of eATP after wounding an *Arabidopsis* leaf with the tip of a micropipette was found to be in the range of 25–45 μM at the injury site (Song et al. 2006).

Another mechanism of ATP release is exocytosis. The human protein VNUT (SLC17A9) was identified as a vesicular ATP transporter loading nucleotides into

secretory vesicles (Sawada et al. 2008). Accordingly, short bursts of ATP discharge were detected in animal cell culture after the fusion of vesicles with the plasma membrane (Zhang et al. 2008). The same release mechanism can also be presumed in plant cells. High concentrations of eATP were imaged at the tips of growing root hairs (Kim et al. 2006). Plant tip growth is characterized by heightened secretion. Consequently, the addition of secretion inhibitors diminished the eATP concentration at the growth tip (Kim et al. 2006).

2.1 Multidrug Resistance Transporters

Multidrug resistance (MDR) transporters belong to the superfamily of ATP-binding cassette proteins and reside in the plasma membrane. One of the family members, the *MDR1* gene product, also known as P-glycoprotein, was shown to be responsible for the steady release of ATP into the extracellular space. The concentration of ATP in the culture medium of Chinese ovary hamster cells increased threefold when mouse *MDR1* was overexpressed (Abraham et al. 1993). Similarly, the overexpression of the *Arabidopsis MDR1* (=*AtPGP1*) gene in *Arabidopsis* or in the yeast *Saccharomyces cerevisiae* doubled the concentration of eATP (Thomas et al. 2000). Patch clamp experiments confirmed that this MDR transporter has ATP channel activity (Abraham et al. 1993) and serves as a source of eATP.

2.2 The Adenylate Transporter PM-ANT1

A member of the mitochondrial carrier family (MCF) was recently identified as plasma membrane-located nucleotide transporter 1 (PM-ANT1). PM-ANT1 shows high homology to other MCF proteins from *Arabidopsis* such as ER-ANT1 (ER-located ATP/ADP carrier, 33 % identity) and AAC1 (mitochondrial ATP/ADP carrier 1, 30 % identity) (Rieder and Neuhaus 2011; Leroch et al. 2008; Klingenberg 2008). Furthermore, PM-ANT1 contains the so-called nucleotide carrier signature, unique for MCF-type ATP/ADP carriers (Rieder and Neuhaus 2011). After heterologous expression of *PM-ANT1* in *E. coli* the import of radioactively labeled ATP or ADP could be demonstrated. The apparent affinities for ATP and ADP transport were 284 μM and 422 μM, respectively (Rieder and Neuhaus 2011). In order to answer the question whether PM-ANT1 facilitates ATP import or export from the cell in vivo, corresponding studies were performed with RNAi (RNA interference) mutants. For this, pollen which represent cells with a naturally high *PM-ANT1* expression were chosen. It was found that pollen with reduced *PM-ANT1* expression contained substantially higher intracellular ATP levels whereas ATP export was decreased (Rieder and Neuhaus 2011). From this it was assumed that PM-ANT1 contributes to ATP export during pollen maturation (Rieder and Neuhaus 2011). PM-ANT1 RNAi mutants are characterized by problems in anther dehiscence leading to reduced pollen liberation and reduced seed numbers (Rieder and Neuhaus 2011).

3 Extracellular ATP

ATP is a highly suitable signaling molecule for intercellular communication: It is small, cell-specific, water-soluble, and abundant in cells [mM range, e.g., 0.9–1.9 mM in *Vicia faba* guard cells (Blatt 1987) and 0.1–0.4 mM in *Vicia faba* embryos (Borisjuk et al. 2003)]. It had been established as a signaling molecule in animals for decades before it became recognized in plants as well at the turn of this century.

3.1 Stimuli for ATP Release

Mechanical stimulation such as shaking (Jeter et al. 2004), touch (Weerasinghe et al. 2009), or osmotic stress (Jeter et al. 2004; Dark et al. 2011) triggers ATP release in plants. Other known stimuli are biotic and abiotic factors. The plant hormone abscisic acid (Clark et al. 2011; Dark et al. 2011), elicitors (Kim et al. 2006; Wu et al. 2008), glutamate (Dark et al. 2011), mycotoxin beauvericin (Srobarova et al. 2009), and ATP itself (Kim et al. 2006) were all shown to prompt ATP discharge. The same holds true for light stimuli (Clark et al. 2011) and salt stress (Dark et al. 2011).

3.2 Cellular Sources

Technically, any cell can release ATP, but growing cells stand out: Shoot apices as well as the tips of roots and root hairs emit more ATP than nongrowing cells (Kim et al. 2006; Weerasinghe et al. 2009). The elevated level of eATP near growth points was observed in several plant species (*Arabidopsis thaliana*, *Lotus japonicus*, *Medicago truncatula*, *Triticum aestivum*) (Kim et al. 2006). More recently, guard cells were also shown to release significantly more ATP than other leaf epidermal cells while opening or closing the stomatal pore (Clark et al. 2011). Guard cells generally contain more cytosolic ATP than other epidermal cells. This was elegantly demonstrated by stably expressing a Förster resonance energy transfer-based indicator of cytosolic ATP in *Arabidopsis* plants which visualized the differences in relative ATP content between guard cells and pavement cells in the epidermis of leaves (Hatsugai et al. 2012). It would be interesting to investigate if other cell types with a prominent ATP release are characterized by a high cytosolic ATP content.

The pronounced expression of the *Arabidopsis* ATP transporter *PM-ANT1* in young siliques, pollen grains, the style, and the phloem of developing leaves and roots suggests that these cell types might also export more ATP than others (Rieder and Neuhaus 2011).

3.3 Physiological Effects

The physiological effects of exogenous application of ATP have been extensively studied. Observed effects included the stimulation of potassium uptake in maize (*Zea mays*) leaves (Lüttge et al. 1974), stimulation of nuclease activity and breakdown of chlorophyll in oat (*Avena sativa)* leaves (Udvardy and Farkas 1973), and faster closure of mechanically stimulated Venus flytraps (*Dionaea muscipula*) (Jaffe 1973). However, one must keep in mind that ATP chelates divalent ions (Sun et al. 2012). Therefore, responses that are dependent on the presence of Ca^{2+} and Mg^{2+} are affected by the chelation ability of ATP, as experimentally demonstrated in the case of potassium uptake (Lüttge et al. 1974).

To differentiate between such a secondary effect of ATP and its primary effects, the medium is often supplemented with Ca^{2+}, as in the case of studies on eATP-induced programmed cell death (PCD) (Sun et al. 2012). Here, PCD was initiated in *Populus euphratica* shoot cell culture by 0.5–2 mM ATP.

A role in plant cell viability was also found in other plant species (*Arabidopsis thaliana*, *Zea mays*, *Phaseolus vulgaris*, *Nicotiana tabacum*), however, with an opposite effect than observed in *P. euphratica*. The depletion of eATP led to cell death, but the application of ATP (0.4–1 mM) maintained cell survival (Chivasa et al. 2005).

A regulatory function of eATP was recently discovered in *Arabidopsi*s stomata movements. The addition of eATP (0.1–1 mM) and the poorly hydrolyzable ATP analog ATPγS (adenosine 5′-[γ-thio]triphosphate) (0.3 mM) promoted opening (Hao et al. 2012). However, Clark et al. (2011) observed that low concentrations (5–15 μM) of the ATPγS induced stomatal opening, while high concentrations (200–250 μM) induced closing. So the effective concentration of eATP seems to be very sensitive to differences in experimental conditions.

The observation that low concentrations of eATP have the opposite effect of high concentrations was also found for growth events (Roux and Steinebrunner 2007). External ATP in the 100–250 μM range enhanced hypocotyl elongation, while higher concentrations (2–10 mM) inhibited hypocotyl growth (Tang 2004). No effect on hypocotyl elongation was observed with the application of 1 mM ATP which was confirmed by Tonon et al. (2010).

Another focus of eATP research has been pathogen defense. Some data suggest that eATP elicits a pathogen-defense response (Jeter et al. 2004; Song et al. 2006; Wu et al. 2008). This is indirectly supported by the finding that the saliva of caterpillars contains ATP hydrolyzing enzymes, and this activity suppressed the plant-defense response (Wu et al. 2012). However, other studies pinned eATP as an inhibitor of such a response (Chivasa et al. 2009).

The list of eATP-mediated effects does not end here. High concentrations of extracellular ATP (2–3 mM) and its analog ATPγS (0.3–1 mM) inhibited pollen germination (Steinebrunner et al. 2003; Reichler et al. 2009). ATP in the same concentration range (2 mM) also inhibited auxin transport in *Arabidopsis* roots (Tang et al. 2003). Many growth processes such as pollen tube elongation (Reichler et al. 2009;

Wu et al. 2007) and root gravitropism (Tang et al. 2003) also respond to eATP. ATPγS (250 μM) decreased pollen tube growth to one-third of the untreated control (Reichler et al. 2009) and, instead of growing down, roots grew horizontally in 1 mM ATP-supplemented agar (Tang et al. 2003).

Table 1 summarizes the different physiological responses to eATP mentioned in this section, but leaves out experiments with ATP analogs. Ever since ATP was recognized as a signaling molecule, poorly or non-hydrolyzable ATP analogs have often been used instead or in addition to ATP to suppress its function as an energy source. The literature is not always consistent regarding the effects of these analogs.

Sometimes an analog, at the same concentration, was as effective as ATP (Foresi et al. 2007; Hao et al. 2012; Song et al. 2006; Sun et al. 2012; Tanaka et al. 2010b; Wu and Wu 2008). In other incidents lower (Reichler et al. 2009; Tang et al. 2003) or higher (Demidchik et al. 2009) concentrations of the analog, compared with ATP, were necessary to reach the same effects. In one case, the analog even triggered opposite events than ATP (Chivasa et al. 2005), and in another case the analog had no effect at all (Kim et al. 2006).

The significance of eATP (Fig. 1) for plant physiology is indisputable, but the underlying modes of action, e.g., signaling, chelation of divalent ions, and provision of energy, obviously need further study to understand the diverse and sometimes conflicting observations.

3.4 Receptors

In mammalians, ATP is recognized by P2 receptors, a family of integral plasma membrane receptors. The four nucleotides ATP, ADP, UTP, and UDP are the primary ligands, but non-hydrolyzable analogs such as ATPγS also serve as agonists.

P2 receptors are divided into two classes: P2X and P2Y. P2X receptors form channels consisting of homo- and heterotrimers which will open after binding the nucleotide ligand and release cations (Na^+, K^+, Ca^{2+}). P2Y receptors are G-protein coupled receptors with seven transmembrane (TM) domains. The ATP signal is transduced by interaction of the receptor with the Gα subunit of the intracellular G-protein heterotrimer [reviewed in Ralevic and Burnstock (1998)].

The first proposition of the existence of purinergic receptors in plants was made after experiments in which ATP caused dose-dependent membrane depolarizations of root hair cells (Lew and Dearnaley 2000). More specific experimental evidence later demonstrated that ATP signaling in plants is mediated through G-coupled receptors (Tanaka et al. 2010b; Hao et al. 2012; Weerasinghe et al. 2009), although no P2Y receptor has been identified in planta so far. The most recent data mining approach of proteome databases of *Arabidopsis thaliana*, *Oryza sativa*, and *Populus trichocarpa* produced several hits, and seven *Arabidopsis* candidates were confirmed to couple with the G-protein subunit 1 in an in vitro protein–protein interaction assay (Gookin et al. 2008).

Searches for candidate P2X genes in *Arabidopsis*, rice, and potato revealed no homologs (Fountain et al. 2008). However, a P2X receptor (CAL54489.1) was found

Table 1 Physiological effects by exogenous application of ATP

Physiological effect[a]	Plant species and tissue	Applied ATP (mM)	Proposed mechanism	Reference
Increase in nuclease activity	*A. sativa* excised leaves	0.5–2	–[b]	Udvardy and Farkas (1973)
Breakdown of chlorophyll		2		
Faster closure of mechanically stimulated Venus flytraps	*D. muscipula* flytraps	0.1	Source of energy	Jaffe (1973)
Stimulation of K^+ uptake	*Z. mays* leaf slices and bundle sheaths	1–2.5	Chelation of Ca^{2+} and Mg^{2+} by ATP	Lüttge et al. (1974)
Membrane depolarization	*A. thaliana* root hairs	0.1–1	Signal or sensor	Lew and Dearnaley (2000)
Elevation of cytosolic Ca^{2+} levels	*A. thaliana* roots	3×10^{-4}–1	Signal[b]	Demidchik et al. (2003)
Inhibition of pollen germination	*A. thaliana* pollen	2–4	Signal[c]	Steinebrunner et al. (2003)
Inhibition of basipetal auxin transport	*A. thaliana; Z. mays* roots	1–3	Inhibition of MDR transporter activity or signal[b,c]	Tang et al. (2003)
Reduction in gravitropic bending	*A. thaliana* roots	1–5	Inhibition of auxin transport[b,c]	
Increase of stress-responsive transcripts	*A. thaliana* cell culture	0.5	Signal[b,c] (for P2-like receptors) in wound, stress, and pathogen responses	Jeter et al. (2004)
Stimulation of hypocotyl growth	*A. thaliana* seedlings	0.1–0.25	Signal[b,c]	Tang (2004)
Inhibition of hypocotyl growth		2–10		
Accumulation of superoxide	*A. thaliana* leaves	5×10^{-4}–0.1	Signal[b] (for P2-like receptors) in wound, stress and pathogen responses	Song et al. (2006)
Increase of stress-responsive transcripts	Seedlings			
Production of reactive oxygen species	*Medicago truncatula* root hairs	1	Signal	Kim et al. (2006)

Accumulation of hydrogen peroxide Production of nitric oxide Increase of intracellular Ca^{2+} levels	*S. miltiorrhiza* hairy root culture	0.01–0.5 0.01–0.5 0.01–0.2	Signal[b] (for P2-like receptors)	Wu and Wu (2008)
Accumulation of hydrogen peroxide Increase in medium pH	*S. miltiorrhiza* hairy root culture	0.01–0.1 0.02–0.1	Signal[b] (for P2-like receptors) in pathogen defense	Wu et al. (2008)
Production of nitric oxide	*Solanum lycopersicum* suspension cells	0.01–5	Signal[b] (for P2-like receptors)	Foresi et al. (2007)
Increase of intracellular Ca^{2+} levels Opening of plasma membrane Ca^{2+} channels Accumulation of superoxide	*A. thaliana* epidermal root protoplasts *A. thaliana* roots	0.01–0.1 0.02–0.03 0.01–1	Signal[b,c]	Demidchik et al. (2009)
Reduction of salicylic acid levels Prevention of pathogen-induced systemic acquired resistance	*Nicotiana tabacum* leaves	0.8 0.01–0.05	Negative regulator of defensive signal transduction	Chivasa et al. (2009)
Production of phosphatidic acid Production of nitric oxide	*S. lycopersicum* suspension cells	0.1 and 1 0.1–1	Signal[b]	Sueldo et al. (2010)
Increase of intracellular Ca^{2+} levels	*A. thaliana* seedlings	0.01–0.5	Signal[b]	Tanaka et al. (2010b)
Programmed cell death	*P. euphratica* shoot cell culture	0.5–2	Signal (for P2-like receptors[c])	Sun et al. (2012)
Stomata opening	*A. thaliana* leaves	0.1–1	Signal[b] (for P2Y-like receptors)	Hao et al. (2012)

[a]Effects were sorted in chronological order of publication year
[b]Source of energy as a mechanism was excluded
[c]Chelation as a mechanism was excluded

in the green alga *Ostreococcus tauri*, which is an evolutionary predecessor of photosynthetic plants (Derelle et al. 2006). This P2X receptor has a 28 % identity to human P2X receptors. The addition of 100 μM ATP to human embryonic kidney cells 293 expressing *OtP2X* evoked channel openings that were not observed in untransfected cells. Nonetheless, it was not possible to demonstrate channel functionality directly in the *O. tauri* plasma membrane (Fountain et al. 2008).

Even though P2 receptors have not been discovered in higher plants, known animal P2 receptor antagonists such as PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid), RB2 (Reactive Blue 2), and suramin (8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulfonic acid) have successfully inhibited eATP-mediated responses in plants (Clark et al. 2010a, b, 2011; Demidchik et al. 2003, 2009; Foresi et al. 2007; Jeter et al. 2004; Song et al. 2006; Sun et al. 2012; Tanaka et al. 2010b; Torres et al. 2008; Tonon et al. 2010; Wu et al. 2008; Wu and Wu 2008). These observations strongly suggest that receptors structurally related to mammalian P2 receptors also exist in plants (Fig. 1).

3.5 Downstream Signaling Events

The first downstream effect of eATP signaling is a biphasic and dose-dependent transient increase in the cytosolic Ca^{2+} concentration $[Ca^{2+}]_{cyt}$ which passes in less than 2 min. This response was attributed to the activation of calcium channels located in the plasma membrane because the addition of calcium channel blockers lowered the eATP-mediated Ca^{2+} peak (Demidchik et al. 2003; Jeter et al. 2004; Tanaka et al. 2010b). The elevation of $[Ca^{2+}]_{cyt}$ activates plasma membrane NADPH oxidases which catalyze the formation of extracellular reactive oxygen species (ROS). The resulting oxidative burst also opens plasma membrane hyperpolarization-activated Ca^{2+} channels amplifying the original eATP signal [summarized in Demidchik et al. (2009) and Tanaka et al. (2010a)].

More recent experiments in *Arabidopsis* root cell protoplasts, however, indicate that the primary elevation in $[Ca^{2+}]_{cyt}$ is not due to a Ca^{2+} flux from the outside of the cell, but rather through the Ca^{2+} release from intracellular Ca^{2+} stores (vacuole, endoplasmic reticulum, mitochondria) (Demidchik et al. 2009).

The development of Förster resonance energy transfer-based genetically encoded Ca^{2+} sensors enabled a high-resolution analysis of the eATP-induced Ca^{2+} spikes (Krebs et al. 2012). Adding ATP to the bathing solution of *Arabidopsis* seedlings triggered nonsynchronous Ca^{2+} waves at the plasma membrane, the cytosol, and the nuclei in epidermal root cells. Interestingly, these oscillations were even nonsynchronous within individual cells.

Another confirmed second messenger of eATP signaling is nitric oxide (NO) (Clark et al. 2010b, 2011; Foresi et al. 2007; Reichler et al. 2009; Tonon et al. 2010; Torres et al. 2008; Wu and Wu 2008). While experiments with mutants deficient in NADPH oxidases and/or NO biosynthesis genes show that both ROS and NO are essential members of the ATP signaling pathway (Clark et al. 2010b, 2011; Demidchik et al. 2009; Reichler et al. 2009; Song et al. 2006; Hao et al. 2012),

the signaling order of the two second messengers remains unclear. Pharmacological studies with hairy root cultures of *Salvia miltiorrhiza* place NO upstream of ROS. The same studies found NO formation upstream and downstream of the eATP-induced intracellular Ca^{2+} elevation (Wu and Wu 2008), suggesting a signal amplification mechanism similar to the one described above for ROS.

In addition to ROS and NO being confirmed transducers of the eATP signal, phosphatidic acid has been observed as another second messenger. Phospholipids which are known downstream effectors of eATP signaling in animals (Ralevic and Burnstock 1998) also triggered oxidative bursts in tomato suspension cultures (Sueldo et al. 2010).

Several genes were identified as targets of the eATP signaling cascade. Not surprisingly, the transcript levels of NADPH oxidase genes such as *Arabidopsis* respiratory burst oxidase homolog D (*AtrbohD*) and the ROS-induced ammonia-lyase gene 1 (*PAL1*) went up (Kim et al. 2009; Song et al. 2006). Increased transcript amounts were also found for members of the mitogen-activated protein kinase family (Demidchik et al. 2009; Jeter et al. 2004; Kim et al. 2009; Song et al. 2006) which is part of stress-responsive signaling cascades. The transcript levels of genes of the ethylene biosynthesis and response pathway were also stimulated by eATP (Jeter et al. 2004; Kim et al. 2009; Demidchik et al. 2009). Similarly, transcripts of lipoxygenase 2 (*LOX2*), a key enzyme in jasmonic acid biosynthesis, were elevated in Northern blot analysis in eATP-treated samples (Song et al. 2006). Both plant hormones ethylene and jasmonic acid are involved in stress and defense responses.

4 Metabolism of Nucleotides and Nucleosides in the Apoplast

4.1 Acid Phosphatases

As the name implies, acid phosphatases require a low pH for optimal activity. Therefore, they are well-fitted enzymes for the acidic apoplast where they release inorganic phosphate from nucleoside tri-, di-, and monophosphates. One member of this large protein family, the purple acid phosphatase PAP3 from *Phaseolus vulgaris*, was cloned and further characterized (Liang et al. 2010). The majority of the protein was found on the plasma membrane (probably anchored by its TM domain), but a fraction of the protein pool also appeared in the apoplast as a soluble protein. Of all the substrates tested, the activity was highest with ATP, making this nucleotide the proposed in vivo substrate (Fig. 1). *PvPAP3* was upregulated upon phosphate starvation, suggesting that its activity helps provide inorganic phosphate in the apoplast for uptake. However, a role in quenching eATP signals is possible as well. In *Arabidopsis*, there is one highly similar protein (NP_178297 NCBI) to PvPAP3 as revealed by phylogenetic tree analysis (Liang et al. 2010).

4.2 E-NTPDases

The term E(cto)-NTPDases was introduced to describe enzymes that cleave a wide range of nucleoside tri- and diphosphates and are active outside the cytoplasm (Zimmermann et al. 2000). Outside, or "ecto," includes the extracellular space as well as the lumen of the Golgi or the endoplasmic reticulum. Historically, NTPDases have been referred to as apyrases, a name originally created by Meyerhof (1945). However, on a quest for a more informative and unifying nomenclature, it was proposed to reserve this name for intra-organellar E-NTPDases (Zimmermann et al. 2000).

In mammalians, the family members NTPDase 1, 2, 3, and 8 were identified as proteins quenching eATP signals [reviewed in Kukulski et al. (2005) and Knowles (2011)]. All four are characterized by two TM domains, one near the N- and one near the C-terminus, inserted into the plasma membrane.

The NTPDases AtAPY1 and AtAPY2 emerged as candidates as their functional homologs in *Arabidopsis* a few years ago. Song et al. (2006) observed that ROS production triggered by eATP was less pronounced in transgenic plants overexpressing *AtAPY2*. This result suggested that AtAPY2 was degrading eATP. One year later, Wu et al. (2007) published that antibodies directed against AtAPY1 inhibited the apyrase activity in the growth medium of *Arabidopsis* pollen tubes. The pollen tubes were releasing eATP, and the amount of released eATP was reduced after the addition of anti-AtAPY1. These results implied that AtAPY1 also was an eATP-degrading enzyme. The same observation was made in cotton fibers, which accumulated more eATP after addition of anti-AtAPY1 to the cotton fiber media (Clark et al. 2010a). The two cotton apyrase candidate proteins GsAPY1 and GsAPY2 are over 65 % identical to AtAPY1 and AtAPY2, making recognition by anti-AtAPY1 likely.

In a different approach, *Arabidopsis* seedlings were exposed to hypertonic stress which led to elevated eATP concentrations (Kim et al. 2009). The increase in eATP levels correlated with enhanced expression of *AtAPY1* and *AtAPY2*. After peak expression levels, the eATP concentrations dropped again. These correlations pointed again to AtAPY1 and AtAPY2 as regulators of [eATP].

The application of the anti-AtAPY1 antibody, which should raise eATP levels, led to the same physiological effect of stomata closure as the addition of 200 μM or 250 μM poorly hydrolyzable ATP (Clark et al. 2011). Here, it was shown that adding the antibody affected not only eATP levels, but also an eATP-mediated signaling event, corroborating its proposed role in purinergic signaling.

However, some conflicting observations arose. While the functional disruption of the *AtAPY1* and *AtAPY2* genes inhibited growth of all cell types (Wolf et al. 2007; Wu et al. 2007), the direct application of exogenous apyrase to root hairs stopped their growth (Kim et al. 2006). The opposite effect, a boost of root hair growth by apyrase activity, had been expected based on the outcome of the genetic knockout experiments.

Another finding contradicting an extracellular function came from a proteomic study which identified AtAPY2 as a Golgi membrane protein (Dunkley et al. 2006).

Recently, considerable doubt was cast on the involvement of AtAPY1 and AtAPY2 in eATP signaling when three groups independently provided evidence for a functional role in the Golgi apparatus. First, AtAPY1 and AtAPY2 were localized to the Golgi (Chiu et al. 2012; Parsons et al. 2012; Schiller et al. 2012). Second, the substrate specificity analysis of AtAPY1 did not include ATP and ADP, but rather the nucleotides GDP, IDP, and UDP (Schiller et al. 2012), which are typical substrates of Golgi apyrases. The enzymatic activity as a GDPase/UDPase was confirmed for AtAPY1 as well as AtAPY2 (Chiu et al. 2012). Third, AtAPY1 or AtAPY2 could rescue glycosylation defects of the yeast mutant *Δgda1* (Chiu et al. 2012; Parsons et al. 2012). GDA1 (guanosine diphosphatase 1) is a Golgi-resident apyrase which is critical for N-glycosylation (Abeijon et al. 1993).

Ironically, the recent data show that the name "apyrase," which was chosen for historical reasons, is functionally correct.

Nevertheless, there is still accumulated evidence that NTPDase activity terminates eATP signaling events in plants (Kim et al. 2006; Tanaka et al. 2010b; Hao et al. 2012; Wu et al. 2007) like it does in animals. For example, the exogenous application of NTPDase in a stomatal aperture assay significantly inhibited the eATP-mediated stomata opening effect (Hao et al. 2012).

The challenge is to find the true plant orthologs of mammalian NTPDases 1–3 and 8. In potato (*Solanum tuberosum*) and cowpea (*Vigna sinensis*), apoplastic NTPDases [StAPY3 (Riewe et al. 2008) and VsNTPase1 (Takahashi et al. 2006)] have been identified with the help of GFP fusion proteins. Western blot analyses of plasma membrane fractions from pea (*Pisum sativum*) and soy bean (*Glycine soja*) also suggested the presence of NTPDases (Day et al. 2000; Thomas et al. 1999).

In *Arabidopsis*, a genetic homology search of the public database TAIR reveals seven NTPDase candidates. One of the corresponding proteins, AAC32915.2, is predicted to have two typical TM domains and is very similar (37–40 %) to the four human NTPDases with extracellular activity. Based on the structural and sequence homology, this protein is a very good start for the search.

4.3 Generation of Adenosine and Adenine in the Apoplast

AMP can be converted to adenosine by phosphatases like purple acid phosphatases mentioned before (PvPAP3, 4.1), although the activity for AMP as a substrate was shown to be rather low (Liang et al. 2010). Typically, 5′ nucleotidases are acting in degradation of AMP to adenosine. These enzymes have been studied intensively in mammals and structures have been resolved (reviewed in Zimmermann et al. 2012). Chen and Kristopeit (1981) were able to partially purify two cytosolic 5′ nucleotidases from wheat germ which accepted AMP and the cytokinin precursor isopentenyl AMP as substrate. However, more detailed work on these enzymes in plants is missing so far.

Adenosine can be deribosylated to adenine by nucleoside hydrolases (NSH) (Fig. 1) which form a small gene family in *Arabidopsis*. Besides cytosolic forms of these enzymes (Jung et al. 2009; Riegler et al. 2011) corresponding activities were

also found in the apoplast of potato tubers and *Arabidopsis* leaves, respectively (Riewe et al. 2008; Jung et al. 2011). The gene encoding latter activity (*NSH3*) was induced after wounding and jasmonic acid treatment indicating a function in wound and pathogen response (Jung et al. 2011). Like adenosine the nucleobase adenine which is one product of NSH3 activity can be reimported into the cell by nucleobase transport proteins (Fig. 1).

5 Nucleoside Transport

In contrast to most nucleotide transporters (ATP/ADP exchangers) nucleoside transport proteins reside in the plasma membrane of cells. Only one member of the plant nucleoside transporter family, ENT1, from *Arabidopsis* was reported to reside in a different membrane, namely the vacuolar membrane, the tonoplast (Fig. 1) (Bernard et al. 2011). Nucleoside transport across the plasma membrane serves in distributing nucleosides between cells and tissues. For example, fully developed cells with mainly structural functions and reduced metabolic activity or cells undergoing senescence can export excess nucleosides as breakdown products from the translational machinery (ribosomal RNA). Via the phloem these nucleosides can then be delivered to growing tissues. Nucleosides have been identified in the phloem sap and supplementation of *Arabidopsis* seedlings with nucleosides was found to be beneficial especially for nitrogen-dependent metabolic pathways such as RNA and amino acid synthesis. In good agreement with these facts is the identification of *ENT* expression in vascular tissues for *Arabidopsis ENT3* (Cornelius et al. 2012) and rice *ENT2* (Hirose et al. 2005).

Additionally, nucleosides can be taken up from the soil in reasonable amounts which may even exceed transport rates determined for amino acids (Cornelius et al. 2012; Hirner et al. 2006). As stable degradation products from nucleic acids nucleosides can be found in soils at quite high amounts (Phillips et al. 1997). The capability of the *Arabidopsis* root system to take up exogenously provided nucleosides was demonstrated accordingly (Traub et al. 2007).

5.1 Nucleoside Transporters of the ENT Family in Humans

The proteins facilitating nucleoside uptake in plants belong to the equilibrative nucleoside transporter (ENT) family also known as SLC29 family (Baldwin et al. 2003). Members of this family were first identified in mammals. Human ENT1 (hENT1; Table 2) was characterized at the molecular level after expression in *Xenopus* oocytes (Griffiths et al. 1997). The proteins hENT1 and hENT2 transport purine and pyrimidine nucleosides with medium to low affinities (Table 2) (Griffiths et al. 1997; Crawford et al. 1998; Visser et al. 2005) and show ubiquitous tissue distribution (Zhang et al. 2007; Griffiths et al. 1997; King et al. 2006). Human nucleoside transport proteins have been identified as important determinants in cancer treatment with nucleoside analog drugs. For example, absence of hENT1 was found to be associated with reduced survival in

Table 2 Characteristics of human, plant, and yeast equilibrative nucleoside transporters

Protein (Accession)	Permeant	K_M (μM)	Subcellular localization	Expression profile	Homology to closest relative within this table	Proposed function
hENT1 (AAC51103)	Uridine Cytidine Adenosine Inosine Guanosine	44.1, Y[1] 234.0, Y[1] 17.8, Y[1] 28.5, Y[1] 47.5, Y[1]	PM[1,13] ER[1,14] MT[1,13] NE[14]	Ubiquitous tissue distribution, including erythrocytes, heart, and CNS[1,12,16]	47 % hENT2	Transport of nucleosides and nucleoside anticancer drugs. Balancing apoplastic nucleoside concentrations
hENT2 (AAC39526)	Uridine Adenosine Inosine	195.0, Y[1] 106.0, Y[1] 180.0, Y[1]	PM ER[14] NE[14]	Ubiquitous tissue distribution, abundant in skeletal muscle	47 % hENT1	Transport of nucleosides and nucleoside anticancer drugs. Balancing apoplastic nucleoside concentrations
hENT3 (AF326987)	Uridine Adenosine	2.02, X[2] 1.86, X[2]	LYS[2]	Widely expressed, including lung, heart, liver, and spleen	33 % hENT1	Lysosomal nucleoside recycling, e.g., in macrophages[11]
AtENT1 (At1g70330)	Uridine Cytidine Adenosine	3.9, Y[1] 30.0, Y[1] 3.6, Y[1]	VAC [7]	Nearly constitutively expressed High in pollen[7]	28 % AtENT3	Export of nucleosides from vacuole (from RNA decay)
AtENT3 (At4g05120)	Uridine Cytidine Thymidine Adenosine Guanosine	3.2[3] 9.5[4], Y 10.0, Y[4] 2.3, Y[5] 2.9[3] 15.5[4],Y; 12, X[6] 18.0, Y[4]	PM[3,7]	Vasculature of leaves and roots[9]	91 % AtENT6	Nucleoside uptake into cells for salvage and degradation (liberation of N), long-distance transport[6, 9]
AtENT6 (At4g05110)	Uridine Cytidine Adenosine Guanosine IPR tZR	6.4, Y[4] 21.2 Y[4] 3.0 Y[4] 11.5 Y[4] 17.0 Y[8] 630.0 Y[8]	PM[4]	Root leaf and flower vasculatures, stomata[8]	91 % AtENT3	Putatively uptake of nucleosides into cells, transport of cytokinin ribosides, long-distance transport[4,8]

(continued)

Table 2 (continued)

Protein (Accession)	Permeant	K_M (μM)	Subcellular localization	Expression profile	Homology to closest relative within this table	Proposed function
OsENT2 (Os07g37100)	Uridine Adenosine IPR tZR	0.7, Y[10] 3.0, Y[10] 32.0, Y[10] 660.0, Y[10]	N.A.	Root vasculature, phloem of leaves, scutellum of germinating seed[10]	66 % AtENT3	Nucleoside uptake for salvage Transport of cytokinin ribosides
FUN26 (AAC04935)	Uridine, Cytidine Thymidine, Adenosine, Inosine[15]	N.A.	VAC[15]	–	22 % AtENT1	Transport of nucleosides across vacuolar membrane[15] May transport nicotinamide riboside[17]

Extracted from [1]Zhang et al. (2007), [2]Baldwin et al. (2003), [3]Li et al. (2003), [4]Wormit et al. (2004), [5]Chen et al. (2006), [6]Traub et al. (2007), [7]Bernard et al. (2011), [8]Hirose et al. (2008), [9]Cornelius et al. (2012), [10]Hirose et al. (2005), [11]Hsu et al. (2012), [12]King et al. (2006), [13]Lai et al. (2004), [14]Mani et al. (1998), [15]Vickers et al. (2000), [16]Griffiths et al. (1997), [17]Lu and Lin (2011)

X expressed in *Xenopus oocytes*, *Y* in yeast, *N.A* not analyzed, *PM* plasma membrane, *ER* endoplasmic reticulum, *MT* mitochondrion, *NE* nuclear envelope, *LYS* lysosome, *VAC* vacuole

patients with gemcitabine (a nucleoside analog and drug)-treated pancreas adenocarcinoma (Marechal et al. 2009). Human ENT1 resides in the plasma membrane and in addition in the nuclear membrane, in the ER membrane, and in the mitochondria (King et al. 2006; Lai et al. 2004; Mani et al. 1998) (Table 2). A special feature of hENT1 is furthermore its high sensitivity towards nitrobenzylmercaptopurine ribonucleoside (NBMPR) which inhibits hENT1-mediated nucleoside transport with K_i values of 0.4–2 nM (King et al. 2006; Griffiths et al. 1997). In contrast, all plant ENT proteins analyzed so far are insensitive towards this inhibitor (Möhlmann et al. 2001; Wormit et al. 2004; Li et al. 2003; Hirose et al. 2005). Knockout mice for *ENT1* are viable and studies with these models have established an important role for ENT in regulating ethanol intoxication (Choi et al. 2004).

5.2 *Nucleoside Transporters of the ENT Family in Plants*

The main nucleoside transport activity in young *Arabidopsis* plants (seedlings) as well as in leaves and roots of fully developed plants was identified with AtENT3 (Fig. 1, Table 2) (Traub et al. 2007; Chen et al. 2006). This protein was characterized biochemically after heterologous expression in yeast (*Saccharomyces cerevisiae*) and *Xenopus* oocytes. AtENT3 transports a broad range of nucleosides with high affinities ranging from 2.3 μM for thymidine to 18 μM for guanosine (Wormit et al. 2004; Li et al. 2003; Chen et al. 2006). The apparent affinity of adenosine transport of 12 μM established by electrophysiological measurements using oocytes expressing *AtENT3* (Traub et al. 2007) is within the range of those reported in yeast (2.9 μM and 15.5 μM, respectively) (Li et al. 2003; Wormit et al. 2004). In contrast to hENT1, AtENT3, like all other plant ENT-type transporters, is not sensitive to NBMPR. In addition, AtENT3 was shown to function as a nucleoside proton symporter (Traub et al. 2007). Like human ENT proteins, plant ENTs, especially AtENT3, were shown to transport nucleoside analogs like 5-fluorouridine or 2-chloroadenosine after heterologous expression in yeast cells as well as in *Arabidopsis* mutants (Traub et al. 2007; Jung and Möhlmann, unpublished results). Such toxic nucleoside analogs have also been proven useful to identify and characterize other mutants in nucleoside metabolism and transport (Schmidt et al. 2004; Jung et al. 2009; Mansfield et al. 2009; Wu and King 1994).

AtENT1 (Fig. 1; Table 2) was the first plant nucleoside transporter to be identified and characterized (Li and Wang 2000; Möhlmann et al. 2001). The biochemical properties of AtENT1 and AtENT3 are quite similar both transport a range of nucleosides and deoxy nucleosides at high affinity (Möhlmann et al. 2001; Li et al. 2003). A high sensitivity of transport towards low concentration of the uncoupler CCCP (m-chlorophenylhydrazone) in yeast cells points to a nucleoside–proton symport mode of transport similar to AtENT3 (Möhlmann et al. 2001). Recently, AtENT1 was identified to reside in the vacuolar membrane (tonoplast), mediating export of nucleosides. Latter property was shown by reconstituting vacuolar membranes from wild-type and *AtENT1* knockdown and overexpression mutants in liposomes and subsequent radioactive transport studies (Bernard et al. 2011).

Arabidopsis mutants with reduced *AtENT1* transcript levels are characterized by reduced activities of cytosolic salvage pathway enzymes and accordingly reduced levels of internal as well as external ATP in pollen, a tissue where *AtENT1* is highly expressed. In contrast, mutants overexpressing *AtENT1* showed increased salvage pathway activity (Bernard et al. 2011; Girke and Möhlmann, unpublished results) (Fig. 1). Furthermore, evidence was generated that the source for vacuolar nucleosides is degradation of vacuolar RNA (Bernard et al. 2011). The presence of RNA oligonucleotides and corresponding degradation activity in vacuoles from tomato suspension cultures had been described before (Abel et al. 1990). Recently it could be shown that autophagy is a mechanism leading to the accumulation of RNA in vacuoles and the Ribonuclease 2 (RNS2) represents an important determinant in the recycling of cellular (most probably ribosomal) RNA (Hillwig et al. 2011).

Interestingly, human and mouse ENT3 (Table 2) are also residing in organelles exhibiting a function in RNA degradation, namely lysosomes (Baldwin et al. 2005). *ENT3* knockout mice were described to develop spontaneous and progressive macrophage-dominated histocytosis. These animals were characterized by spontaneous lymphadenopathy (swelling of lymph nodes) and splenomegaly (enlargement of the spleen) accompanied by a significant shorter life span compared to littermates (Hsu et al. 2012). The identified increased nucleoside levels in lysosomes of macrophages were accompanied by an increased intralysosomal pH leading to altered macrophage function in *ENT3* knockout mice. Thereby, macrophages function in phagocytosis and degradation of the majority of dying cells and their nucleic acids (Hsu et al. 2012). In this respect, the functions of AtENT1 in the plant vacuole and mouse ENT3 in lysosomes appear to be comparable.

5.3 *Nucleoside Transporters of the ENT Family in Yeast*

Yeast cells express one member of the ENT family, still named FUN26 for "function unknown," although reports have assigned a function to this protein (Vickers et al. 2000; Lu and Lin 2011). FUN26 shares 15–18 % identical amino acids with hENT1, 2, and 3 and 22 % with AtENT1. Thus, AtENT1 has the highest identity to FUN26 compared with all other *Arabidopsis* ENT proteins (Table 2). Apart from the sequence similarity, FUN26 shares the localization in endogenous membranes (prevacuole or vacuole) with hENT3 and AtENT1 (Vickers et al. 2000; Lu and Lin 2011). Another common feature of all three proteins (FUN26, hENT3, and AtENT1) is the presence of an extended (39–45 amino acids) N-terminal sequence compared to their closest relatives. In addition, the hydrophilic N-terminus of hENT3 contains an endosomal/lysosomal targeting motif (Baldwin et al. 2005). Uptake of radioactively labeled cytidine, adenosine, and uridine into *FUN26* expressing *Xenopus* oocytes indicates that FUN26 is a functional member of the nucleoside transporter family ENT (Vickers et al. 2000). In addition a function in nicotinamide riboside (NmR) release from vacuoles under conditions of phosphate starvation was adjudicated to FUN26 (Lu and Lin 2011). Vacuolar NmR is derived from

nicotinamide nucleotide (NMN) by cleavage of the phosphate group by Pho8 phosphatase (Lu and Lin 2011).

5.4 Cytokinin Riboside Transport by Plant ENT Members

Cytokinins are plant phytohormones that regulate a multitude of processes in plant development and growth. Examples are the regulation of the cell cycle, stimulation of shoot meristems, counteraction of leaf senescence, and their function in plant adaptation to environmental stress (Kieber 2002; Ha et al. 2012). The synthesis of cytokinins starts from ADP, ATP, or tRNA by isopentenylation of the adenine moiety (Ha et al. 2012). Therefore, cytokinins are structurally similar to purine compounds. It is therefore not surprising that several enzymes within purine metabolism also exhibit the capacity to convert cytokinin derivatives. Examples are 5′ nucleotidase from wheat germ, adenine phosphoribosyltransferase from *Arabidopsis* and *Physcomitrella patens* and nucleoside hydrolase 1 from *Arabidopsis* (Chen and Kristopeit 1981; Allen et al. 2002; Moffatt et al. 1991; Jung et al. 2009; von Schwartzenberg et al. 1998). Furthermore, proteins able to transport cytokinin bases and ribosides were identified after heterologous expression in yeast cells. These proteins belong to the family of purine permeases (PUP) and ENTs (Bürkle et al. 2003; Hirose et al. 2005, 2008; Sun et al. 2005). The apparent affinity of *Arabidopsis* PUP1 for trans-zeatin is reported to be 40 μM. *Arabidopsis* ENT6 transports isopentenyladenine riboside (iPR) with an apparent affinity of 17 μM and the corresponding K_M value for trans-zeatin riboside (tZR) was 630 μM, respectively (Bürkle et al. 2003; Hirose et al. 2008). Similar results were obtained for rice ENT2; the K_M values were 660 μM (tZR) and 32 μM (iPR) (Hirose et al. 2005). These transport systems suffer from relatively low affinities towards cytokinins given that measured cytokinin concentrations are far below these values. For example, in the xylem of *Urtica dioica*, cytokinins were found in the low nanomolar range, and in tomato xylem, about 170 nM trans zeatin ribosides were reported, respectively (Beck 1996; Albacete et al. 2008). However, by using *Arabidopsis* cell culture cells low-affinity transport of cytokinin bases was observed supporting the idea that purine permeases may be involved in cytokinin uptake (Cedzich et al. 2008). Concerning transport of cytokinin ribosides, the overexpression of *OsENT2*, a proven cytokinin transporter from rice (Hirose et al. 2005), in *Arabidopsis* driven by a constitutive promoter did not result in any obvious developmental or growth responses (Bernard and Möhlmann, unpublished results). Thus, no direct evidence for a participation of PUP or ENT proteins in cytokinin metabolism in planta has been generated so far. The lack of cytokinin typical growth responses in *PUP* or *ENT* mutants might be due to the high redundancy of these transport proteins; however, it is also possible that the correct transport systems for cytokinin bases and ribosides have not yet been identified.

6 Conclusion and Perspective

Many components of an eATP signaling transduction pathway have been identified in plants, including downstream signaling events and a transporter delivering the eATP. The nature of the enzymes quenching the signal is emerging, but the most important link, the identification of a receptor for ATP in the plasma membrane of higher plants, remains missing.

Various plant nucleoside transporters have been identified and characterized. However, still some of the *Arabidopsis* ENT proteins await clarification of their biochemical and physiological functions. Of special interest is the complete unraveling of the function of endogenous proteins (AtENT1) and the question whether ENT-type transporters contribute to cytokinin metabolism at the physiological level.

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Gene and Protein Identifiers

AtAPY1 (At3g04080), *AtAPY2* (At5g18280), *AtPGP1* (At2g36910), *AtGPA1* (At2g26300), AtrbohD (AF055357), *GDA1* (856669), GhAPY1 (GU385147), GhAPY2 (GU385148), human E-NTPDase 1 (NC_000010.10), human E-NTPDase 2, human E-NTPDase 3, human E-NTPDase 8, LOX2 (L23968), *OtP2X* (Ot07g02160), PAL1 (NM129260), *PM-ANT1* (At5g56450), PvPAP3 (D2D4J4 UniProtKB), StAPY3 (EU125183), VsNTPase1 (Q5NT85 UniProtKB), hENT1 (AAC51103), hENT2 (AAC39526), hENT3 (AF326987), *AtENT1* (At1g70330), *AtENT3* (At4g05120), *AtENT6* (At4g05110), *OsENT2* (Os07g37100), FUN26 (AAC04935).

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Regulation of PPi Levels Through the Vacuolar Membrane H^+-Pyrophosphatase

Ali Ferjani, Shoji Segami, Mariko Asaoka, and Masayoshi Maeshima

Contents

Abstract Inorganic pyrophosphate (PPi) is a high-energy compound, although the free energy change of its hydrolysis is approximately 60 % that of ATP. PPi is generated as a by-product of macromolecule biosyntheses in plants, especially in proliferating cells. In living cells, the accumulation of PPi causes the suppression of these metabolic processes and the formation of insoluble Ca–PPi complexes. To avoid these negative effects, the vacuolar H^+-pyrophosphatase (H^+-PPase)

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hydrolyzes PPi and pumps H^+ across the vacuolar membrane to maintain their acidic state. Importantly, recent studies on *fugu5*, the H^+-PPase loss-of-function mutants, have clearly demonstrated that their phenotype is rescued by the expression of the yeast cytosolic PPase IPP1, which hydrolyzes cytosolic PPi but has no effect on vacuolar acidification, thus strongly suggesting that the role of the H^+-PPase lies in the consumption of the inhibitory PPi rather than vacuolar acidification. In this chapter we describe the chemical properties and metabolic role of PPi, in addition to the physiological functions of H^+-PPase and soluble PPase revealed by using several mutant lines.

Keywords Arabidopsis • H^+-PPase • PPi homeostasis • Proton pump • Metabolism • Germination • Gluconeogenesis • Sucrose • IPP1 • Vacuolar pH • Storage lipid mobilization • Leaf development • Cotyledon • Cell proliferation • Cell expansion • Compensation • *fugu5* mutants

1 Introduction: PPi in Plant Cells

Living organisms have evolved metabolic networks that enable efficient interplay between energy-producing and energy-consuming reactions for survival and propagation of their offspring. In cells, whether simple or complex, the manner by which energy-rich molecules are made and consumed is basically conserved. In eukaryotes, ATP can be produced by a number of distinct cellular processes that generate energy, most of which take place in the mitochondria and the chloroplasts.

Inorganic pyrophosphate (PPi; diphosphate) is known as a biological high-energy compound because its standard free energy of hydrolysis is comparable to that of ATP. However, the importance of PPi has been obscured by ATP. In living cells, PPi is principally produced by hydrolysis of ATP. For example, biosyntheses of macromolecules, such as DNA, RNA, and proteins, generate PPi as a by-product of these reactions that use ATP or other nucleotide triphosphates such as GTP. Hydrolysis of PPi by cytosolic soluble pyrophosphatases (sPPases) and membrane-bound H^+-translocating pyrophosphatases (H^+-PPases) produces inorganic phosphate, which is reused for generation of ATP, other nucleotides, and phosphate compounds. Therefore, the relationship of ATP and PPi is that of light and shadow.

The scientific literature on the biological role of PPi published from the 1940s to the end of the year 1999 has been well reviewed and critically evaluated (Heinonen 2001). About 195 known biochemical reactions that produce PPi are catalogued into different categories, most of them being fundamentally important for cell life (Maeshima 2000; Heinonen 2001).

PPi has been proposed to provide a pyrophosphate bond as an energy donor instead of ATP during the origin of life on Earth (Baltcheffsky and Baltcheffsky 1992). Indeed, H^+-PPase utilizes the pyrophosphate bond of PPi instead of ATP to

perform active transport of protons across the membrane. This PPi-dependent enzymatic activity was first reported for the photosynthetic bacterium *Rhodospirillum rubrum* in 1967 (Baltcheffsky 1967), and for plant vacuolar membrane fractions in the middle of the 1980s (Chanson et al. 1985; Rea and Poole 1986). Enzymes were then identified in mung bean (*Vigna radiata*) (Maeshima and Yoshida 1989), *R. rubrum* (Nore et al. 1991), and other plants (for review, see Maeshima 2000; Gaxiola et al. 2007). H^+-PPase has attracted biochemists and plant scientists at viewpoints of novel energy transducing proton pump and as a regulator of PPi level in plant cells.

The concentration of PPi directly affects the chemical equilibrium of macromolecule biosyntheses and other PPi-generating metabolic processes such as fatty acid β-oxidation in cells. Indeed, a high concentration of PPi stops the DNA polymerization reaction. In cells, the PPi level is maintained as a balance between the generation and hydrolysis of PPi. Several biosynthetic reactions, which are catalyzed by, for example, aminoacyl-tRNA synthetase, RNA polymerase, and fatty acyl-CoA synthetase, generate PPi, and some enzymes, including H^+-PPase, consume PPi (Heinonen 2001). How is the PPi level maintained by these enzymes? What is the physiological importance of PPi homeostasis in plant cells? PPi is notable as an energy donor comparable to ATP, a regulator of metabolism, and an effector of cotyledon morphogenesis as described later.

Here, we focus on PPi in plants and describe the physicochemical and biochemical properties of PPi, the metabolic processes of generation and hydrolysis of PPi, and the physiological relevance of the maintenance of PPi levels in cells.

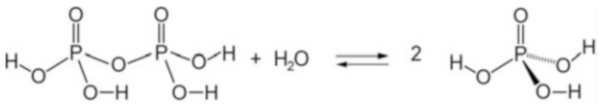
2 Physicochemistry of PPi

2.1 Biochemistry of PPi Hydrolysis and Synthesis

PPi can be chemically prepared by heating orthophosphoric acid, thus producing pyrophosphate (*pyro* from the Greek, meaning "fire"). In living cells, PPi is generated mainly from hydrolysis of ATP and other nucleotide triphosphates, which are used as phosphate donors in the biosyntheses of DNA, RNA, polypeptides, glycogen, and cellulose. Displacement reactions of ATP with R-O (R, chemical moiety or residue) can be categorized into three types: (1) phosphoryl transfer (produces R-O-Pi and ADP), (2) pyrophosphoryl transfer (R-O-PPi and AMP), and (3) adenylyl transfer (R-O-Pi-O-ribose-adenine and PPi). Macromolecule polymerization processes include the above reaction (3) and produce PPi in cells.

Next we'll describe the free energy change of hydrolysis and synthesis of PPi. The standard free energy of PPi hydrolysis is −19.2 kJ/mol (Frey and Arabshahi 1995), which is smaller than that of ATP (−30.5 kJ/mol). Under physiological conditions the value becomes higher. For example, the actual free energy change of hydrolysis under physiological conditions of 2 mM Pi and 0.20 mM PPi at 25 °C is calculated to be −28.9 kJ/mol. Cytosolic PPi concentrations have been reported to

be 0.1–0.3 mM in plants (Weiner et al. 1987; Stitt 1989; Takeshige et al. 1988). When PPi is used by H^+-PPase, the free energy from hydrolysis of PPi is converted to active translocation of protons across the membrane. If PPi is hydrolyzed by a soluble type PPase, the energy is released as heat in cells.



How can H^+-PPase generate a pH gradient across membranes? Here we calculate a pH gradient using the above free energy values with the following equation:

$$\Delta G_t = RT\,2.30\log(C_2/C_1) + ZF\Delta\Psi,$$

where ΔG_t is the free energy change for transport, R is the gas constant (8.315 J/mol K), T is the absolute temperature (here 298 K), C_1 is the concentration of H^+ in the space 1, C_2 is the concentration of H^+ in the space 2, Z is the charge on the ion (1 for H^+), F is the Faraday constant (96,480 J/V mol), and $\Delta\psi$ is the transmembrane electrical potential (V). The value of log (C_2/C_1) means the pH gradient that is generated by consuming the free energy from hydrolysis of the high-energy compounds. Here we assume that $\Delta\psi$ of the vacuolar membrane is 0.060 V. Theoretically, PPi hydrolysis has the potential to generate a pH gradient of 4.1 pH units calculated from the actual free energy change under physiological conditions. In other words, H^+-PPase has the theoretical capacity to acidify vacuoles down to pH 3.1 under physiological conditions with the assumption of cytosolic pH 7.2.

The accumulation of crystals of calcium pyrophosphate (Ca_2PPi) is known as a rheumatologic disorder in human connective tissues. Indeed, Ca_2PPi is practically insoluble in water. This may be one of the reasons why PPi cannot become the main energy currency in living cells instead of ATP. In plant cells, Ca_2PPi complexes are negligible under normal physiological conditions, because the concentrations of Ca^{2+} and PPi in the cytosol are kept at around 0.1 mM and 0.1−0.3 mM, respectively. In plant cells, some PPi may exist in an Mg_2PPi complex.

2.2 *Quantification of PPi in Cells and Tissues*

A few research groups have focused on the PPi concentration, which has been calculated to be at 0.1–0.3 mM in the plant cell cytosol. High concentrations of PPi increase the risk of formation of Ca–PPi complexes, which are insoluble even in cells as described above. PPi has been reported to exist predominantly in the cytosol in plants as mentioned above, and if PPi were to accumulate in vacuoles, it might easily form insoluble Ca_2PPi complexes.

In growing hypocotyls of mung bean, the PPi concentration is at the range of 64–84 nmol/g fr wt (Maeshima 1990). The cytosolic PPi level is around 0.2 mM with the assumption that the cytosol occupies about 30 % of the total cell volume, including the cell wall space. In other plants and other tissues, PPi levels are comparatively lower than those of mung bean hypocotyls (for review, see Heinonen 2001). It is expected that the level is higher in growing cells that actively synthesize macromolecules. In etiolated young seedlings of *Arabidopsis*, the PPi level has been reported to be 51 nmol/g fr wt (Ferjani et al. 2011). If the cytosol occupies 30 % of the seedling cells, the cytosolic PPi concentration is calculated to be 0.17 mM. What effects do cytosolic PPi concentrations have on PPi-utilizing enzymes? H^+-PPase can express its full activity only at more than 0.1 mM as demonstrated by patch clamp analysis of mung bean H^+-PPase expressed in yeast (Nakanishi et al. 2003). The mung bean enzyme has a K_m for the substrate Mg_2PPi, an actual form of the substrate, of 4.8 μM (Nakanishi et al. 2003). This value means 18 μM PPi with Mg^{2+} at 1 mM.

Several biochemical methods have been used to determine the PPi concentration in tissue extracts. Researchers have used three approaches to ensure accurate PPi quantification: (1) stop the biosynthesis and hydrolysis of PPi immediately during or after tissue homogenization, (2) prepare the PPi fraction with high recovery, and (3) measure the PPi concentration with sensitive methods. In most cases, tissues are frozen in liquid nitrogen and homogenized in chilled 80 % ethanol (Ferjani et al. 2011) or 0.45 N perchloric acid (Takeshige and Tazawa 1989; Maeshima 1990) to prevent enzyme reactions. After centrifugation or gel filtration of the tissue extract, the soluble fractions are used for PPi measurements, such as in enzyme-based methods, liquid chromatography, or gas chromatography. Also, H^+-PPase can be used for PPi quantification theoretically. A critical point of this method is to remove Ca^{2+} and Pi from the tissue extracts, because these ions partially inhibit H^+-PPase. In addition to recent advances in PPi quantification, we need new methods to determine or monitor PPi levels in real time in living cells to understand the physiological relevance of PPi homeostasis.

3 PPi in Metabolism

3.1 PPi Producing Processes

In 1941, Cori found that PPi accumulated in rat liver extracts, which represents the first report about PPi formation in a biological system (see Cori et al. 1951). Later, in 1948, Kornberg described the first biological reaction that led to PPi production, which he named pyrophosphorolysis with reference to the well-known phosphorolysis (Kornberg 1948). In 1957, he proposed that pyrophosphorylases mostly act in the direction of PPi production favoring the formation of several stable biochemical compounds (Kornberg 1957). He stated later in 1962 that coupling of hydrolysis of

PPi by inorganic PPase makes these reactions practically irreversible, a hypothesis that is now widely accepted (Kornberg 1962). Then, a larger number of pyrophosphorylases were reported in the early 1960s that were subdivided into different groups based on the type of reactions they catalyze (Imsande and Handler 1961). It is now generally accepted that most PPi comes from hydrolysis of nucleotide triphosphates (NTPs) in vivo.

In plant cells, PPi is a by-product of biosynthetic processes characteristic of actively growing cells, such as macromolecule biosyntheses and ß-oxidation of fatty acids (Maeshima 2000; Heinonen 2001). Nucleic acid syntheses (polymerization of DNA and RNA), their subsequent modifications such as polyadenylation and capping of mRNA, and pyrimidine and purine nucleotides syntheses de novo are all sources of PPi (Heinonen 2001). The biosyntheses of amino acids, such as histidine and tryptophan, aminoacyl-tRNA (for protein synthesis), and their modifications also produce PPi (Heinonen 2001). Of course, besides all the reactions presented so far, the hydrolysis of NTPs (as stated above), and the synthesis of cyclic nucleotides (cNMP) by the action of adenylate cyclase, liberates PPi in a stoichiometric manner. The activation of fatty acids (FAs, below) by a long chain acyl-CoA synthetase (LACS) is usually coupled to the hydrolysis of ATP to AMP and PPi, and generates acyl-CoAs (Fulda et al. 2004). In plants, syntheses of starch and sucrose (Suc, below) go through an ADPG pyrophosphorylase (AGPase, below) reaction, where one PPi is formed for each glucose unit incorporated (Stitt et al. 1985).

In C_4 plants, a very high rate of PPi production is expected to occur. Oxaloacetate, produced in the reaction between PEP and CO_2, catalyzed by PEP carboxylase, is then transferred to the bundle sheath cells, where CO_2 is released to be used in carbon assimilation (Edwards and Huber 1981). PEP regeneration in the mesophyll chloroplasts by pyruvate orthophosphate dikinase (PPDK) releases one PPi for each CO_2 assimilated. However, this high PPi production in the mesophyll chloroplasts is counterbalanced by their high PPase activity, which is at least one order of magnitude lower in C_3 plants, and is likely to be an adaptation to C_4 photosynthesis (Hatch and Slack 1970).

Gluconeogenesis, on the other hand, is also a "hot spot" for PPi production in plant cytosols in which PPi is generated by the reaction of PPi-dependent phosphofructokinase (PFP, below). The reaction catalyzed by UDPG pyrophosphorylase (UGPase, below) with UTP and Glc-1-P produces UDPG and PPi. Finally, PPi is produced in plastids during the conversion of the Glc-1-P pool to produce ADPG, which is the substrate for starch synthesis, by the action of AGPase.

3.2 PPi-Utilizing Reactions

Plants have four enzymes, PFP, UGPase, PPDK, and H^+-PPase, that can use PPi as a phosphoryl donor. PFP is a cytosolic enzyme, widely distributed in the plant kingdom, that catalyzes a readily reversible reaction between fructose-6-phosphate (Fru-6-P) and fructose-1,6-bisphosphate (Fru-1,6-bisP; Stitt et al. 1982; Carnal and

Black 1983; Kruger et al. 1983; Kubota and Ashihara 1990). Assays in vitro using PFP purified from potato (*Solanum tuberosum*) tubers have demonstrated that Pi inhibits the reaction in the direction of Fru-6-P phosphorylation (glycolysis) and that PPi is inhibitory to the opposite reaction (gluconeogenesis; Stitt 1989).

UGPase acts in both directions producing PPi in the synthesis of and consuming it in the mobilization of Suc. In Suc synthesis it acts together with Suc phosphate synthase and Suc phosphatase. On the other hand, during mobilization of Suc, the first enzyme acting is Suc synthase, which in spite of its name does not make Suc in vivo, but rather breaks it down. It is obvious that the mobilization of Suc by Suc synthase and UGPase saves biochemical energy compared to the alternative invertase–hexokinase pathway (Taiz and Zeiger 2010). PPDK is a well-known enzyme of the C_4 photosynthetic pathway where it catalyzes the ATP- and Pi-dependent formation of PEP, the primary CO_2 acceptor molecule, from pyruvate (Chastain et al. 2002). Finally, the H^+-PPase of the tonoplast and other endomembranes transports protons in vivo at the expense of PPi. These H^+-PPases are the main focus of this review; therefore, they will be introduced in detail later on.

3.3 Deficiency and Excess of PPi

In all animals and many microbes, the large amount of cytosolic PPi that is generated as a by-product of anabolism in actively proliferating cells is thought to be tightly regulated through immediate hydrolysis by abundant inorganic PPases in a highly exergonic reaction (Kornberg 1962; Josse and Wong 1971; Maeshima 2000; Heinonen 2001). In plants, the plastids contain very high PPase activity (Gross and ap Rees 1986; Weiner et al. 1987) and very low PPi levels (Weiner et al. 1987). In contrast, in the plant cytosol, PPi is not wasted because there is little or no PPase activity (allowing a significant pool of PPi to accumulate), and PPi-dependent enzymes exist that can use PPi instead of ATP to maintain several cellular activities (Weiner et al. 1987).

Treatments of plant tissues in vitro using inhibitors, or the overexpression of some key enzymes that consume PPi, have in extreme cases resulted in a several-fold change of PPi levels. For example, the incubation of spinach leaf discs with imidodiphosphate (IDP), a potential inhibitor of the vacuolar H^+-PPase, but not of PFP, raises PPi levels up to fivefold (Neuhaus and Stitt 1991). This led to the conclusion that H^+-PPase plays an important role in the removal of PPi from the cytosol.

In contrast, the overexpression of PPase from *Escherichia coli* in potato and tobacco plants leads to a twofold increase in total PPase activity, which is comparable with that normally found in the chloroplast (Sonnewald 1992). PPi contents were later measured in the same transgenic potato tubers and estimated to be half that of the wild type (Geigenberger et al. 1998). In conclusion, introducing a cytosolic PPase into transgenic plants results in an alteration in photoassimilate partitioning that seems to be shifted towards soluble sugar accumulation

(Sonnewald 1992). Finally, transgenic sugarcane clones (*Saccharum* spp. hybrids) with varying degrees of reduced PFP activity display no visible phenotypical changes, but significant changes are evident in their metabolite profiles. In fact, decreased PFP leads to a reduction of PPi levels in older internodes, consistent with PFP catalyzing a net gluconeogenic (PPi-generating) flux in aged internodes (van der Merwe et al. 2010). In summary, all these studies point to a robust interaction between PPi levels and cellular metabolism. Yet, despite all these efforts, direct evidence of the role of H^+-PPase in vivo is still missing, leaving our understanding about this important issue fragmentary and speculative.

4 PPi Hydrolysis by H^+-PPase

4.1 H^+-PPase

H^+-PPase is a key enzyme that regulates the PPi balance in the cytosol. This has been clearly demonstrated by loss-of-function mutants of H^+-PPase as described in the next section (Ferjani et al. 2011). Here, we overview the H^+-PPases in plants. In addition to its role as a scavenger of PPi, H^+-PPase has a role in proton pumping across the vacuolar membrane of plants as the fourth proton pump. Plants have the mitochondrial and chloroplast F-type ATPase, the plasma membrane-type H^+-ATPase (P-type ATPase), and the vacuolar-type H^+-ATPase (V-type ATPase) (Gaxiola et al. 2007; Martinoia et al. 2007). The F-type ATPase functions as an ATP synthetase in mitochondria or chloroplasts, and the P-type ATPase functions in plasma membranes to acidify the extracellular space and maintain cytosolic pH. Indeed, the pH optimum of plasma membrane ATPases is relatively acidic at pH 6.5–7.0 (Faraday and Spanswick 1992). This means that ATPases efflux H^+ from the cytosol across the plasma membrane when the cytosolic pH decreases. The third H^+-ATPase, V-ATPase, acidifies the vacuoles and maintains the cytosolic pH together with H^+-PPase. The V-ATPase is also located in the Golgi apparatus, although its main localization is the vacuolar membrane.

The fourth H^+ pump, H^+-PPase, has several characteristic features: (1) the enzyme consists of a single polypeptide of approximately 80 kDa; (2) its substrate is the unique, simple, and high-energy compound PPi; and (3) H^+-PPase is found in a limited number of organisms, such as plants and several photosynthetic bacteria, and is not found in yeast or animal cells (Maeshima 2000). Point three above might be tightly linked to the specific physiological properties of plants, especially the PPi balance in cells, as described in this chapter. For point one above, the membrane topology of mung bean H^+-PPase has been investigated (Mimura et al. 2004; Nakanishi et al. 2001), and recently a clear crystal structure of the enzyme has been solved (Lin et al. 2012). The tertiary structure of plant H^+-PPase shows a high similarity with Na^+-pumping PPase (Na^+-PPase) of a hyperthermophilic bacteria *Thermotoga maritime*, which is a sodium pump.

Most plants have two types of H^+-PPases, I and II, which differ in their primary sequence and K^+ dependence of enzyme function (Drozdowicz et al. 2000). The type I H^+-PPase (At1g15690) requires K^+ at more than 30 mM for maximal activity and functions in vacuolar membranes (Maeshima and Yoshida 1989). The type II enzyme (At1g78920 and At1g16780), which does not require K^+, is localized in the Golgi apparatus and related vesicles (Segami et al. 2010). In *Arabidopsis*, there is no difference in the molecular activities of the type I and II enzymes, and the protein amount of the type II is less than 0.2 % of the type I (vacuolar H^+-PPase). Thus, only the vacuolar H^+-PPase should be considered in understanding PPi homeostasis in plant tissues.

4.2 Soluble PPases

It is impossible to understand the physiological regulation of PPi levels in plant cells without considering soluble PPases (sPPases) as well as H^+-PPases. For a long time, it was thought that the activities of sPPases are negligible in plant cells (Weiner et al. 1987). In addition to vacuolar H^+-PPase, however, cDNAs of sPPases (EC 3.6.1.1) have been cloned from green algae and land plants (Kieber and Signer 1991; Rojas-Beltrán et al. 1999; Gómez-García et al. 2006). *Arabidopsis* has six sPPase genes: *AtPPa1* (At1g01050), *AtPPa2* (At2g18230), *AtPPa3* (At2g46860), *AtPPa4* (At3g53620), *AtPPa5* (At4g01480), and *AtPPa6* (At5g09650). These homologues are divided into two groups: prokaryotic (AtPPa1 to AtPPa5) and eukaryotic types (AtPPa6) (Rojas-Beltrán et al. 1999; Gómez-García et al. 2006). Both types share common properties with the vacuolar H^+-PPase, such as the requirement of Mg^{2+} for activity. The two types, however, exhibit differences in molecular size and tertiary structure. For example, the yeast enzyme (eukaryotic type) is larger than the *E. coli* enzyme (prokaryotic type) and functions as a homodimer (Salminen et al. 2002), while the *E. coli* enzyme forms a homohexamer (Avaeva et al 1999).

AtPPa6 has a cleavable transit peptide and localizes to the chloroplast stroma (Schulze et al. 2004). For the *Chlamydomonas reinhardtii* enzyme (Cr-sPPase I, eukaryotic type), its K_m for PPi is 10.5 μM and K_{cat} 315 s^{-1} (Gómez-García et al. 2006). The K_m is very small when compared with H^+-PPases and other sPPases. The *AtPPa6* gene is induced by Glc, Fru, and Suc in *Arabidopsis*. Therefore, these plastid-type (eukaryotic type) sPPases are thought to enhance the reaction of AGPase, which generates PPi and stimulates starch biosynthesis (Schulze et al. 2004; Gómez-García et al. 2006).

For prokaryotic types, AtPPa1 and AtPPa4 expressed in *E. coli* have been used for enzymatic characterization. These two isoforms have similar molecular masses of approximately 24 kDa and have similar kinetic properties: AtPPa1 (K_m for PPi, 114 μM; k_{cat}, 9.28 s^{-1}) and AtPPa4 (K_m, 101 μM; k_{cat}, 9.20 s^{-1}) (Sancha et al. 2007). AtPPa1 is expressed in most tissues (Sancha et al. 2007), consistent with information from open databases such as Genevestigator (http://www.genevestigator.ethz.ch/).

As for the phenotypic properties when *AtPPa1* is overexpressed in seeds, a decrease in the amount of stored lipids and an increase in the levels of free sugar and starch have been reported (Meyer et al. 2012). In mutants of *AtPPa1* and *AtPPa4* knocked-down by RNAi techniques, the stored lipid content is increased.

sPPase is involved in the self-incompatibility of *Papaver rhoeas* (poppy). In the process of self-incompatibility, a sPPase (Pr-p26.1s) in the cytosol of pollens is inactivated by phosphorylation in response to calcium signaling (de Graaf et al. 2006). As the result of an increase in intracellular PPi levels, macromolecule biosynthesis is inhibited and pollen tube growth is suppressed. This prevents self-fertilization. In *Arabidopsis*, which is a self-compatible plant, the vacuolar H^+-PPase exists in pollen at relatively high levels, but loss-of-function mutant plants show normal self-fertilization (Segami, unpublished data). Therefore, sPPase, which may not be inactivated by phosphorylation, hydrolyzes PPi in the cytosol of H^+-PPase knockout plants (*AVP1/VHP1;1*) even during fertilization. Furthermore, recent reports suggest that sPPases are involved in the recycling of phosphate. A member (AtPPsPase1) of the haloacid dehydrogenase superfamily possesses pyrophosphatase activity and is induced under phosphate-deficient conditions in *Arabidopsis* (May et al. 2011).

The activity of sPPases in the cytosol was thought to be very low and negligible for a long time. At present, we need detailed information on the total and individual activities of sPPases and on the physiological changes of their activities for understanding their physiological roles and contributions when compared with H^+-PPases.

5 PPi Balance in Seedling Development

In this section, we will briefly introduce the isolation of H^+-PPase loss-of-function *fugu5* mutants of *A. thaliana*, and the analyses that led to the discovery of previously unrecognized important roles of this enzyme in plant growth and development.

5.1 Compensation and Plant Development

Animal and plant forms display a spectacular diversity that is familiar to everyone; however, how their organ sizes are predetermined has been a long-standing issue in biological research (Conlon and Raff 1999; Tsukaya 2008). Which factors, genetic pathways, and developmental mechanisms promote the progression of organ growth and then restrict it when appropriate organ size is reached? Is size a function of proliferative growth of single cells and their final size, or is it rather under developmental programs acting organ-wide? In order to deepen our knowledge of coordinated organ-size control in multicellular organisms, answers to these questions and other closely related issues are eagerly awaited.

Intrinsic regulatory mechanisms inscribed in the plant's genome orchestrate developmental programs that when properly executed determine the appropriate size of an organ, though this is significantly affected by various environmental cues, such as light, temperature, water, etc. (Tsukaya 2005). Owing to their determinate fate, leaves have the potential to grow for only a defined period of time; hence, their final size could be interpreted as a simple function of the number and size of component cells. Importantly, an organ-wide control system has been suggested by the so-called "compensation" phenomenon, in which a decrease in cell number, caused by a mutation that compromises cell cycling, triggers excessive cell enlargement (Tsukaya 2002, 2006, 2008; Beemster et al. 2003; Horiguchi et al. 2006a; Ferjani et al. 2007, 2008, 2010; Micol 2009; Kawade et al. 2010; Horiguchi and Tsukaya 2011). Based on the above observations, non-cell-autonomous regulation should be assumed as fundamentally important for proper organogenesis. As such, compensation-exhibiting mutants represent excellent models in which the coordinated regulation between organ-size determinants (i.e., cell number and size) is abolished. Cloning the mutated genes and analyzing their functions and contribution to developing organs is a promising approach for solving the long-standing size regulation enigma.

Five decades have passed since the earliest report about compensation (Haber 1962). Since then, this phenomenon has been reported to occur in transgenics in which the cell cycle is inhibited (Hemerly et al. 1995; De Veylder et al. 2001), and in mutants defective in genes that positively regulate cell cycling (Mizukami and Fischer 2000; Kim and Kende 2004; Horiguchi et al. 2005; Ferjani et al. 2007, 2011). Hence, compensation appears to be a universal phenomenon that is not restricted to the model organism *Arabidopsis thaliana* (Horiguchi and Tsukaya 2011 and Table 1 therein), but also occurs in a wide range of plant species such as *Nicotiana tabacum*, *Oryza sativa*, and *Antirrhinum majus* (Hemerly et al. 1995; Barrôco et al. 2006; Delgado-Benarroch et al. 2009, respectively).

To uncover compensation mechanisms, large-scale screenings have been carried out that have identified several *Arabidopsis* mutants with leaf size and/or shape defects (Horiguchi et al. 2006b). Among them, five mutants that are collectively called *fugu1-fugu5* exhibit typical compensation (Ferjani et al. 2007). For the last decade or so, analyses of the developmental dynamics in a large number of mutants and transgenics that exhibit compensation have been fruitful and have shed light on several key features of the compensation phenomenon (summarized in Horiguchi and Tsukaya 2011).

5.2 *Discovery of* fugu5 *Mutants*

Cloning of the genes mutated in each *fugu* line has been conducted to elucidate their function. Interestingly, among them, *fugu5* mutations have been mapped to *AVP1/VHP1;1*, which encodes for the vacuolar type I H^+-PPase in *Arabidopsis* (Maeshima 2000; Heinonen 2001; Li et al. 2005; Ferjani et al. 2011; Fig. 1b).

Importantly, the *fugu5* defect in the number and size of cells is severe in early leaves, namely, the cotyledons (Fig. 1a) and the first and second foliage leaves, but not in later leaves (Ferjani et al. 2007, 2011). Also, the *fugu5* morphological and cellular phenotypes are completely suppressed by exogenously supplied Suc (Ferjani et al. 2011). In addition, the three *fugu5* mutant alleles harboring different molecular lesions in the *AVP1* locus have no detectable PPi hydrolysis activity (Fig. 1c, d). This total loss of H^+-PPase activity does not affect the activity of the vacuolar H^+-ATPase, indicating that the observed phenotypic defects are H^+-PPase specific (Fig. 1d). These findings strongly suggested a defect in post-germinative cell proliferation in cotyledons that is responsible for the altered cell division and expansion observed in *fugu5*.

In an earlier report by Li et al. (2005), the aberrant shoots and roots of the *avp1-1* mutant (a mutant allele of *fugu5*) have been assumed to be the result of altered auxin distribution due to defective proton pumping. In contrast to this report, application of exogenous auxins (IAA or NAA) and the use of an auxin-responsive *DR5::GUS* reporter construct showed that the defects in *fugu5* do not involve any altered auxin-mediated organ development (Ferjani et al. 2011). A potential explanation of *avp1-1* allele-specific phenotypes is provided elsewhere (Ferjani et al. 2012). Note that the vacuolar pH in *fugu5* is slightly increased by only 0.25 pH units compared to the wild type, demonstrating the relatively small contribution of H^+-PPase to vacuolar acidification when compared to the *vha-a2 vha-a3* double mutant of the V-ATPase, in which vacuolar pH shifted by 0.5 pH units (Ferjani et al. 2011; Krebs et al. 2010). Finally, mobilization of the major seed storage proteins is not affected at all in *fugu5* mutants (Fig. 2a).

AVP1 has a dual molecular function: consumption of PPi and pumping of protons into the vacuoles. Given the broad belief that AVP1 is important as a proton pump, discrepancy among the *avp1-1* and *fugu5* mutant phenotypes has again raised a fundamental question that awaits a clear answer: What is the biological role of H^+-PPase? Which one of the two functions of AVP1 is critical for plant development?

5.3 *H^+-PPase, a Master Regulator of Cytosolic PPi Homeostasis*

In plant cells, the vacuolar H^+-ATPase together with the H^+-PPase is assumed to be responsible for vacuole acidification, and to cooperatively establish a transmembrane proton gradient as the driving force for the transport of solutes (Maeshima 2001; Gaxiola et al. 2007). Although a major role of H^+-PPase as a proton pump has been reported (Li et al. 2005), the importance of the enzymatic hydrolysis of PPi has generated less interest. Therefore, to gain new insights into this fundamental issue, it has been necessary to evaluate the contribution of these two functions separately.

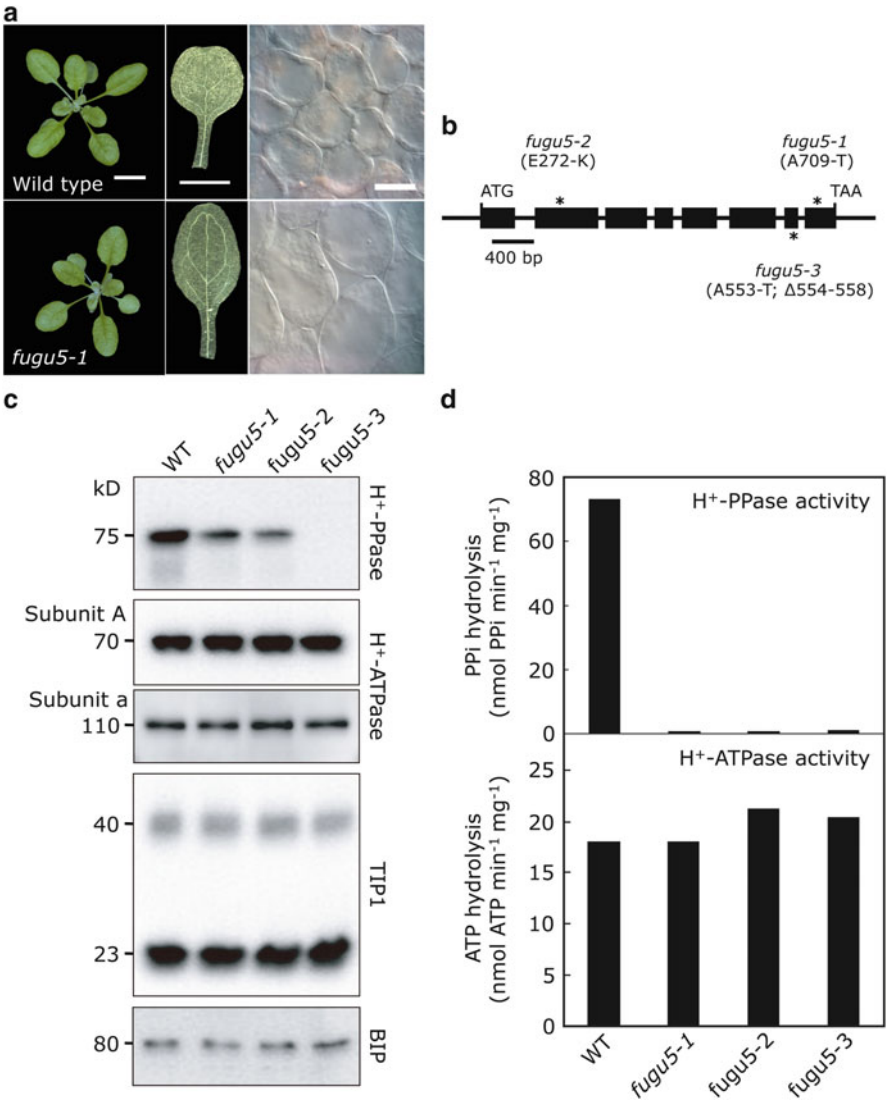


Fig. 1 Morphological and cellular phenotype of *fugu5* mutants. (**a**) Shoots of 25-day-old plants (*left panels*). Bar: 10 mm. Mature cotyledons from 21-day-old plants (*middle panels*). Bar: 2 mm. Micrographs showing palisade cells in the subepidermal layer from a paradermal view (*right panels*). Bar: 50 μm. (**b**) Schematic representation of the *FUGU5/AVP1* gene. Exons are shown as *filled rectangles*. The molecular lesions in each of the three loss-of-function *fugu5* alleles are indicated by an *asterisk*. The Ala^{709} residue is replaced by Thr in *fugu5-1*. The Glu^{272} residue is replaced by Lys in *fugu5-2*. The Ala^{553} residue is replaced by Thr, and five residues, from Leu^{554} to Ala^{558}, are deleted in *fugu5-3*. (**c**) Protein levels of vacuolar membrane proton pumps, BIP, and aquaporin as determined from crude membrane fractions from plant shoots. Apparent molecular weights of the immunostained bands are shown in each panel. H^+-PPase protein was not detected in the *fugu5-3* mutant line. (**d**) Substrate–hydrolysis activity of H^+-PPase (*top*) and H^+-ATPase (*bottom*) (Figure slightly modified with permission from Ferjani et al. (2007, 2011); http://www.plantphysiol.org http://www.plantcell.org, "Copyright American Society of Plant Biologists")

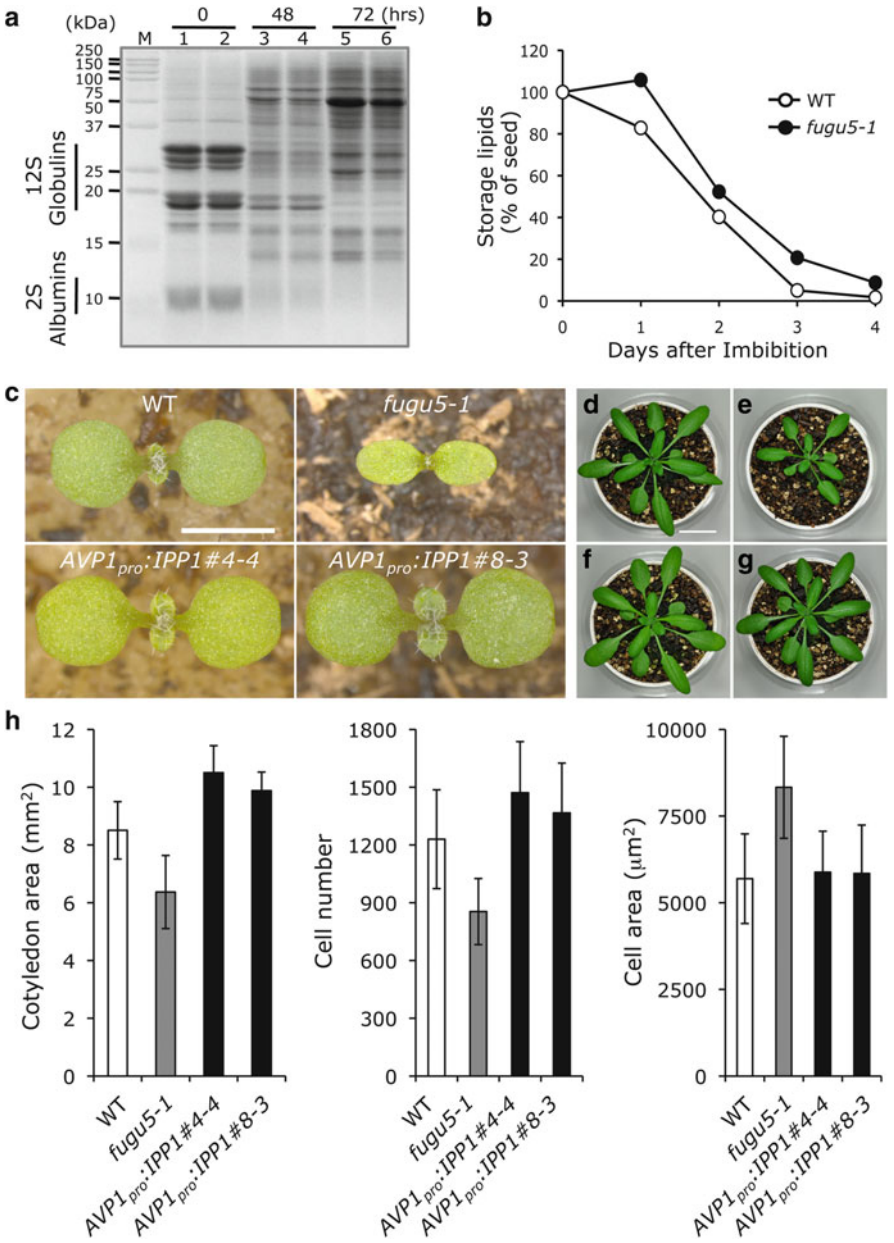


Fig. 2 Yeast cytosolic PPase IPP1 complements the morphological and cellular phenotypes of *fugu5*. (**a**) Effect of H^+-PPase dysfunction on seed storage protein mobilization. Protein from dry seeds (day zero) and seeds imbibed for 1–3 days were extracted, applied to SDS-PAGE, and subsequently stained with Coomassie Brilliant blue. Lane numbers 1, 3, and 5 indicate WT samples. Lane numbers 2, 4, and 6 indicate *fugu5-1* mutant samples. Results were reproducible in three independent experiments. (**b**) Effect of H^+-PPase dysfunction on seed lipid reserve mobilization. The amounts of reserved lipids in WT and *fugu5-1* mutant were determined during post-germinative growth. Samples were prepared from dry seeds (day zero) and etiolated seedlings

To address this, Ferjani and colleagues have used an elegant approach that consists of checking the effect of specific removal of cytosolic PPi alone on *fugu5* mutant phenotypes. For this, they have used the cytosolic PPase IPP1 of *Saccharomyces cerevisiae* that can only hydrolyze PPi, but does not perform proton pumping (Lundin et al. 1991). In doing so, transgenics in the *fugu5* background expressing IPP1 protein (*AVP1$_{pro}$::IPP1*, below) should recover their ability to hydrolyze cytosolic PPi, but still lack the vacuolar H^+-PPase functioning as a pump. In their construct, the AVP1 promoter was used to express IPP1 in a natural leaf-developmental context, avoiding any ambiguous side effects due to ectopic expression. Amazingly, all of the *fugu5* mutant phenotypes recognized so far have been perfectly rescued by complementation with IPP1 (Fig. 2; see also Ferjani et al. 2011 for more details). Moreover, the vacuolar pH in the *AVP1$_{pro}$::IPP1* transgenic lines remains equal to that of *fugu5*, demonstrating that the slight upshift of vacuolar pH has no direct correlation with the observed *fugu5* phenotypes (Ferjani et al. 2011). Taken together, the above empirical evidence indicates that in early seedling development, characterized by an active metabolism, the important role of the H^+-PPase lies in the consumption of the inhibitory PPi rather than vacuolar acidification (Ferjani et al. 2011; Bertoni 2011).

Additionally, *fugu5* displays a short hypocotyl phenotype in absolute darkness that is rescued by Suc supply or in *AVP1$_{pro}$::IPP1* transgenics where PPi has been specifically removed. The Suc-dependent growth feature of *fugu5* phenocopies that of mutants defective in seed storage lipid mobilization (Graham 2008). Whereas triacylglycerol (TAG) lipolysis (Fig. 2b) and FA catabolism via ß-oxidation are unaffected, *fugu5* seedlings accumulate a much higher amount of PPi and less Suc than wild-type plants (Ferjani et al. 2011). This data confirms that gluconeogenesis is partially inhibited by the high cytosolic PPi levels in *fugu5*. Given the broad range of biochemical reactions inhibited by PPi, including gluconeogenesis, this data shows that H^+-PPase maintains a normal leaf cell number and size through PPi hydrolysis (Ferjani et al. 2011). The phenotypes of *fugu5* are specifically rescued upon Suc supply at the onset of seed imbibition, highlighting the heterotrophic nature of growth during the earliest stages of plant development. The fact that such phenotypes are no longer observed in leaves formed at later stages indicates a transition to autotrophic growth in which the leaves are exclusively nourished from photosynthesized carbohydrates.

Fig. 2 (continued) after 1, 2, 3, and 4 days after imbibition (see Ferjani et al. 2011 for details). Data are means from more than three independent experiments. (**c**) Heterologous expression of the *IPP1* gene rescued *fugu5* gross phenotypes. Gross morphology of seedlings of WT, *fugu5-1*, and two representative lines of *AVP1$_{Pro}$:IPP1* transgenic plants at 7 DAS. Scale bar, 2 mm. (**d–g**) Heterologous expression of the *IPP1* gene rescues delayed growth of *fugu5*. Gross morphology of WT, *fugu5-1*, and *AVP1$_{Pro}$:IPP1* #4-4 and *AVP1$_{Pro}$:IPP1* #8-3 transgenic plants, respectively, at 28 DAS. Scale bar, 2 cm. (**h**) Heterologous expression of the *IPP1* gene totally rescues *fugu5* cellular phenotypes. Average area, cell number, and cell size of cotyledons of WT, *fugu5-1*, and two representative lines of *AVP1$_{Pro}$:IPP1* grown on rockwool for 25 DAS are shown. Data are means with standard deviation ($n = 8$). DAS: days after sowing (Figure slightly modified with permission from Ferjani et al. (2011); http://www.plantcell.org, "Copyright American Society of Plant Biologists")

Very recently, Meyer and colleagues reported a dominant low-seed-oil mutant (lo15571) of *Arabidopsis* generated by enhancer tagging in which the conversion of photoassimilates to oil is reduced (Meyer et al. 2012). Immunoblot analysis revealed increased levels of AtPPa1 (At1g01050) protein in developing siliques of lo15571. Interestingly, *AtPPa1* encodes a cytosolic sPPase and is one of five closely related genes that share predicted cytosolic localization (Meyer et al. 2012, see also Sect. 4.2). In their scenario, they emphasized that the rate of cytosolic glycolysis of Suc mobilization, as the major route providing precursors for seed oil biosynthesis, is strongly influenced by the expression of endogenous sPPases (i.e., limited PPi pools), although no data on PPi contents have been provided.

Altogether, these novel findings disagree with the decades-old belief that vacuolar acidification through H^+-PPase is crucial for plant growth. Rather, the overall metabolic context that produces PPi during germination provides a more elaborate view into the role of the vacuolar H^+-PPase. In addition, as described in previous sections, the *Arabidopsis* genome contains two other genes, *AtVHP2;1* and *AtVHP2;2*, encoding for type II enzymes that are exclusively localized in the Golgi apparatus (Mitsuda et al. 2001; Segami et al. 2010, see also Sect. 4.1). The physiological contribution of the type II H^+-PPases in vacuolar acidification and cytosolic PPi hydrolysis is fairly negligible, consistent with the absence of detectable PPi hydrolyzing activity in the total membrane fraction of *fugu5* (Hruz et al. 2008; Winter et al. 2007; Segami et al. 2010; Ferjani et al. 2011, 2012). For these reasons, we should consider AVP1 as the master PPase regulating cytosolic PPi levels. Indeed, this important conclusion represents a milestone for future studies related to PPi homeostasis in plants.

6 Conclusions and Future Prospects

The central role of plant H^+-PPases in PPi homeostasis has been uncovered, but this discovery does not mark the end of the road, which has been circuitous. In fact, many critical questions remain unanswered. First, does our actual understanding of a major role for vacuolar H^+-PPase in PPi homeostasis totally rule out its role as a proton pump? In *vha-a2 vha-a3*, a mutant lacking tonoplast-specific V-ATPase activity, the vacuolar pH is elevated (pH 6.4) but remains significantly more acidic than the cytosol (pH 7.4; Krebs et al. 2010). Also, a lack of the vacuolar H^+-PPase does not significantly affect vacuolar pH (Ferjani et al. 2011). In agreement with these findings, overexpression of the vacuolar H^+-PPase in the *vha-a2 vha-a3* mutant background did not restore their phenotype, and the triple mutants *vha-a2 vha-a3 fugu5-1* are viable (Kriegel et al. ICAR 2012 abstract book). Based on this, a contribution of the endosomal V-ATPase to vacuolar pH has been strongly suggested. Besides, we believe that comparative studies taking advantage of *fugu5* mutants and $AVP1_{pro}$*::IPP1* transgenics together with *vha-a2 vha-a3* should help resolve this intriguing issue. Also, provided that cold and anoxia are two kinds of environmental stresses that can specifically induce H^+-PPase expression, while

V-ATPase is severely inhibited, the potential role of H^+-PPase as a proton pump might be masked as most phenotypes described here were deduced from studies conducted under standard culture conditions.

Second, while huge strides have been made, the target sites of PPi inhibition starting from TAGs seed reserves and ending up with Suc syntheses de novo have yet to be determined. For this, quantitative high-throughput metabolomics should be very useful. Last, the crystal structure of a *V. radiata* H^+-PPase (VrH^+-PPase) complexed with IDP, a non-hydrolyzable PPi analogue, at 2.35 Å resolution has been recently reported (Lin et al. 2012). Given that the amino acid residues involved in PPi binding, hydrolysis, and proton translocation are highly conserved among various plant species, the point mutations in *fugu5-1 and fugu5-2* and the relatively short deletion in *fugu5-3* (Fig. 1) should be useful for comparative analyses of the structure–function relationship between the H^+-PPases in a wide range of other plant species.

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Interactions Between Nutrients and Crassulacean Acid Metabolism

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Contents

Abstract CAM expression is under the control of an intricate signaling network involving numerous interconnected environmental cues strongly linked to nutrient accessibility for plants. Mineral nutrients are essential elements for conferring plant resistance to drought or salinity while both these abiotic stresses can disturb the nutritional relations and modulate the photosynthetic pathway of CAM plants. Hypothetical connections between CAM photosynthesis and mineral nitrogen metabolism have been suggested as possible mechanisms conferring physiological advantages for plant survival under severe environment conditions. Although the mineral nutrition of CAM plants has received relatively scarce attention, some studies have consistently demonstrated that different degrees of nutrient fertilization can influence CAM expression. In addition, the nutritional aspect of CAM regulation is frequently related to the action of other environmental factors, especially light and water availability. Among all the essential macronutrients, variation in nitrogen content usually shows the strongest correlation with CAM activity. Some evidence indicates that specific effects of nitrate and ammonium ions are crucial in the CAM modulation rather than an influence of nitrogen status itself.

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Therefore, future research is required to unravel the effects and the respective metabolic and signaling mechanisms of interaction between mineral nutrients and other environmental signals on CAM photosynthesis.

1 Introduction

Crassulacean acid metabolism (CAM) is a photosynthetic pathway manifested in a diverse range of species and life forms. The phylogenetic and ecological diversity found among CAM plants is also reflected in a remarkable plasticity of the metabolic schemes underpinning net carbon gain (Bhagwat 2005). Although CAM has attracted worldwide interest because of its ecological importance and appealing aspects of comparative biochemistry and physiology, the essential pathways of carbon flow in CAM plants are by far less understood than the photosynthetic modes reported for C_3 and C_4 plants (Winter and Smith 1996; Holtum 2002; Doubnerová and Ryslavá 2011). In addition, the predictable connections between carbon cycle and nutritional metabolism that might occur in CAM plants have also received scarce attention.

Although it is challenging to make generalizations about the patterns of operation and distribution of CAM, plants with higher degree of CAM plasticity usually inhabit unstable environments where abiotic stress is caused by the interaction of multiple factors, such as water and nutrient availability, salinity, and changes in light and temperature (Cushman 2001; Lüttge 2004, 2010; Herrera 2009). Accordingly, CAM is largely associated with plants that live in a wide range of tropical and subtropical habitats that typically harbor exceptional heterogeneity of geosystems spanning all major tropical biomes. Moreover, a large number of CAM species inhabiting tropical rainforests are epiphytes and hemi-epiphytes which are subject to particular problems of water and mineral nutrient supply in this habitat (Zotz and Hietz 2001; Lüttge 2004, 2010; Hughes et al. 2013). Therefore, the adaptive fitness of plants that occupy these highly changeable environments relies on their ability to efficiently perceive and respond to the dynamic network of interacting stressors by expressing metabolic flexibility, as observed in several CAM plants (Lüttge 2010).

Apparently, the most important abiotic stress for CAM modulation concerns water limitation. In fact, it is widely accepted that CAM is an adaptive mechanism that provides significant functional advantage in water economy to plants inhabiting regions characterized by seasonal or intermittent limitations in water availability (Cushman 2001; Lüttge 2002, 2004, 2010; Herrera 2009; Borland et al. 2011). However, water shortage is often accompanied by nutrient deficiency since drought can directly reduce nutrient availability for plants (Chapin 1991). Moreover, studies have consistently shown that inducible CAM can also be modulated in some plants by salinity (Cushman and Bohnert 2004; Cushman et al. 1990; Lüttge 2004), thermoperiodic variation, and low temperatures (Haag-Kerwer et al. 1992; Neales et al. 1980; Nievola et al. 2005), whereas most of these environmental cues can often affect CAM expression by causing osmotic stress due to plant dehydration (Boudsocq and Laurie 2005). Interestingly, it is known that mineral nutrients are essential elements for conferring plant resistance to

drought or salinity. Moreover, both drought and salinity can disturb the nutritional relations through their effects on nutrient availability, transport, and partitioning in plants (Hu and Schmidhalter 2005).

In the light of this intricate network involving environmental control of CAM expression, a complex scenario is evident in which CAM signaling pathways are subject to numerous interconnected environmental cues, including the mineral nutrients, whose uptake, transport, and assimilation are strongly linked to water availability. Therefore, this chapter examines the flexibility of CAM expression in the light of multiple environmental stresses, emphasizing the relationship between nutritional availability and the modulation of CAM pathway. The existing information directs the main focus on examining the plasticity of CAM expression in response to environmental constraints involving nutritional availability (especially nitrogen resources) and the implications of these adaptive responses for plant survival. Finally, we address potential directions for future research in this field.

2 CAM Photosynthesis: Origin and Basic Features

During the central carboxylating process present in all plants, the Calvin and Benson cycle, CO_2 is fixed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), which is responsible for the entry of CO_2 into C_3 cycle through the carboxylation of ribulose-1,5-bisphosphate (Lara and Andreo 2005). However, RUBISCO can also act as an oxygenase adding O_2 (instead of CO_2) to ribulose-1,5-bisphosphate, resulting in loss of the fixed carbon during the photorespiratory carbon oxidation cycle. Photorespiration is not only a wasteful mechanism that leads to a net loss of CO_2, but is also an energy-demanding process that consumes light-generated ATP and NADPH. Such unfavorable oxygenase reaction of RUBISCO is assumed to be a residual consequence of its own evolutionary history since this enzyme evolved when atmospheric CO_2 concentrations were high and oxygen concentrations low. However, when CO_2 decreased and O_2 increased on Earth atmosphere, plants evolved different CO_2 concentrating mechanisms to mitigate photorespiration and, perhaps, the most flexible solution was the CAM photosynthetic syndrome (Doubnerová and Ryslavá 2011; Gowik and Westhoff 2011).

CAM operates on a background of RUBISCO-mediated CO_2 fixation through the engagement of phosphoenolpyruvate carboxylase (PEPC) for nocturnal CO_2 fixation (Borland et al. 2011), PEPC being an enzyme with nearly 60-fold higher affinity towards its substrate than RUBISCO. This elevated affinity seems to be crucial for the highly efficient inorganic carbon acquisition by PEPC during nighttime (Lüttge 2010). In CAM plants these two CO_2-fixating enzymes are found in the same cells and the two processes of CO_2 fixation are temporally separated, thus requiring complex control of alternative metabolic pathways in response to environmental signals (Bhagwat 2005). The multiple origins of CAM indicate that relatively small evolutionary alterations were necessary in the primary

C_3 metabolism for the establishment of this photosynthetic mode. In fact, it is assumed that all metabolic enzymes and transporters necessary for CAM expression are already present in the photosynthesizing cells of all higher plant species. Therefore, the CAM biochemistry can be considered as the result of adjustments and reorganization of the metabolic machinery common in the housekeeping functions of all photosynthesizing plants (Lüttge 2005, 2006; Borland et al. 2011; West-Eberhard et al. 2011). Basically, the net consequence of the CAM pathway for terrestrial plants is that CO_2 is fixed with considerable water economy relative to C_3 photosynthesis, since this process occurs mainly during the nighttime, when there is a lower evaporative demand in the environment and a larger air-leaf CO_2 concentration gradient (Winter and Smith 1996; Herrera 2009).

Accordingly, CAM plants usually close their stomata during most of the daytime period to prevent water loss. During nighttime, the atmospheric CO_2 enters the cytoplasm of photosynthesizing cells where it is converted to HCO_3 by carbonic anhydrase and then to oxaloacetate (OAA) by PEPC using phosphoenolpyruvate (PEP). OAA is subsequently converted to malate by NAD-malate dehydrogenase (MDH), while the malic acid accumulates in large vacuoles causing the nocturnal acidity characteristic of CAM tissues. During the daylight hours, malate is transported back into the cytoplasm, where CO_2 is released by one of the decarboxylating enzymes (malic enzyme, ME or PEP carboxykinase, PEPCK) that can be present in the basic biochemical subtypes of CAM photosynthesis. Subsequently, PEP is regenerated by a pyruvate phosphate dikinase (PPDK) and the released CO_2 behind close stomata enters the chloroplasts where it is refixed by RUBISCO on the conventional C_3 cycle (Vu et al. 2002; Gowik and Westhoff 2011).

One important component of CAM plasticity is that several plants expressing this metabolic syndrome can exhibit a considerable variation in both amplitude of CAM cycle and its contribution to carbon gain (Holtum 2002). In this sense, CAM in plants can be constitutive, facultative, inducible, and reversible, and also variants occur that fix nighttime-respired CO_2 by PEPC without any accompanying nocturnal opening of stomata—the so-called CAM cycling or CAM idling variants (Cushman 2001; Dodd et al. 2002; Edwards and Ogburn 2012). Such versatile expression of CAM photosynthesis is mainly under environmental control, whereas most C_3-CAM facultative plants can switch between these photosynthetic modes and use the CAM option as an adaptive alternative for increasing fitness to environmental dynamics (Lüttge 2010; Winter and Holtum 2011; Edwards and Ogburn 2012).

3 How Can CAM Pathway Influence the Regulation of Nitrogen Metabolism?

Carbon and mineral nutrient (especially nitrogen) metabolisms are strongly connected in almost every biochemical pathway in plants. Therefore, carbon and nutrient sensing and signaling are key mechanisms that enable plants to modulate

their metabolism and development in response to the environmental resources (Coruzzi and Bush 2001; Coruzzi and Zhou 2001). For example, as for the carbon fixation reactions, the first steps of nitrogen assimilation into amino acids also take place in the chloroplasts. Besides, the nitrogen assimilation reactions utilize carbon skeletons from the tricarboxylic acid cycle, which makes these reactions not only crucial for the cycling of carbon within the plant, but also essential for the nitrogen metabolism (Lawlor 2002; Suzuki and Knaff 2005; McAllister et al. 2012). Moreover, it is known that higher levels of atmospheric CO_2 increase both carbon and nitrogen assimilation (Geiger et al. 1998; Leakey et al. 2009), while the reduced expression of RUBISCO can significantly alter carbon and nitrogen metabolisms, diminishing amino acid content (Matt et al. 2002). Additionally, it has been well recognized that the uptake, assimilation, and remobilization of nitrogen are regulated and controlled by photosynthetic rates to a considerable extent (Zheng 2009; McAllister et al. 2012). Despite all this evidence, the impact of photosynthetic carbon fixation adaptations conferred by CAM pathway on nitrogen metabolism and signaling mechanisms has remained largely unexplored, and their potential connections are still elusive (Freschi et al. 2009). Such scenario is even more scarce of information when other nutrients are considered, which impels the main focus of the present discussion on potential links involving CAM expression and nitrogen metabolism.

Some important clues about possible connections between CAM photosynthesis and mineral nitrogen metabolism can be revealed by comparative studies of enzyme isoforms which can participate in both CAM cycle and in housekeeping functions of C_3 plants with direct and/or indirect impacts on nitrogen metabolism. For example, it is known that PEPC plays an important role in nitrogen assimilation, particularly when the main nitrogen source is ammonium (Masumoto et al. 2010; Doubnerová and Ryslavá 2011; McAllister et al. 2012). In fact, ammonium has a central participation connecting nitrogen and carbon metabolisms since this compound, though potentially toxic, represents the reduced form of nitrogen used by plants for nitrogen assimilation into amino acids, proteins, nucleotides, and chlorophylls, among other molecules (Hodges 2002). Whatever ammonium is directly absorbed by plants or being a product of nitrate conversion catalyzed by the sequential action of the nitrate reductase (NR) and the nitrite reductase (NiR), ammonium must be rapidly assimilated into nontoxic organic compounds by the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) in the chloroplasts. Therefore, the proper function of the GS/GOGAT cycle strictly depends on the interaction between nitrogen and carbon metabolisms (Ireland and Lea 1999; Lea and Ireland 1999; Hodges 2002).

This interconnection between carbon and nitrogen metabolisms is particularly maintained due the energetic demand for reduction and assimilation of nitrogen by photosynthesizing tissues since GS activity requires energy (ATP) and GOGAT utilizes reducing power in the form of NADH or reduced ferredoxin. In addition, ferredoxin is also required for the nitrite reduction, while the reducing power for nitrate to nitrite conversion is supplied by NAD(P)H (Ireland and Lea 1999; Lea and Ireland 1999; Hodges 2002; Britto and Kronzucker 2005; Dutilleul et al. 2005).

GOGAT activity also utilizes the tricarboxylic acid cycle intermediate 2-oxoglutarate as carbon skeleton, making the 2-oxoglutarate an additional key metabolite at the crossroads of carbon and nitrogen metabolisms. In fact, 2-oxoglutarate participates in several reactions related to nitrogen recycling, such as in the replenishment of glutamate removed by nitrogen assimilation into amino acids. Moreover, 2-oxoglutarate is also a direct regulator of the PEPC and citrate synthase activities, while both these enzymes participate in the cellular redox control and in the sugar and/or organic acid flux (Hodges 2002; Araújo et al. 2013).

Another interesting example illustrating the potential connections between carbon and nitrogen metabolisms is given by the joint function of some essential enzymes that participate in the concentrating CO_2 process during CAM evolution, such as PEPC, PPDK, and ME, which provide the replenishment of carbon skeletons for the tricarboxylic acid cycle and nitrogen assimilation, thereby contributing to the biosynthesis of amino acids. These enzymes are present in all types of plants and can exhibit housekeeping roles in C_3 plants where they play a wide range of metabolic functions, both under favorable and stressful conditions. For example, increased activities of PEPC, PPDK, and/or ME were found in plants under various types of abiotic stress, such as drought, high salinity, and scarcity of phosphate and iron, among others (Aubry et al. 2011; Doubnerová and Ryslavá 2011; Langdale 2011). Interestingly, most of the environmental stimuli that modulate the non-photosynthetic isoforms of these enzymes in all types of plants are also the main factors modulating their counterparts which play key roles in the photosynthetic machinery during CAM expression. Understanding the role and relationship of these enzymes in the C_3 state is likely to be useful for predicting how these metabolic elements might interact when recruited into the background of CAM pathway. This comparative view becomes even more relevant when particular cases of C_3-CAM facultative species are considered, such as in *Clusia minor*, where a single isoform of PEPC can support both housekeeping functions of the C_3 state and the particular requirements of the CAM photosynthesis (Lüttge 2006).

Furthermore, some points must be highlighted concerning the relatively high degree of conservation observed in some metabolic responses of CAM and C_3 plants subjected to extreme stress conditions. For example, particularly under severe environmental constraints which may limit photosynthesis, C_3 plants usually close their stomata and the internal CO_2 recycling can play an important role of minimizing or preventing respiratory carbon losses, which, in turn, partially ameliorates oxidative damage to plant metabolism. Concurrently, this recycling of respired CO_2 during the night is an essential function of CAM pathway under idling mode since the recapture of respiratory CO_2 can contribute to prevent carbon losses at the expense of plant growth under severe stresses (Herrera 2009; Klavsen et al. 2011).

Accordingly, it is suggested that, under stressful environments, the enzymes PEPC, ME, PPDK, and MDH can form a metabolic cycle that might confer several physiological advantages for C_3 plants exposed to adverse conditions (Doubnerová and Ryslavá 2011). Curiously, the key metabolic reactions that compose this suggested cycle performed by C_3 plants under environmental stresses can

functionally parallel the general operation of these enzymes during the CAM cycle. In accordance with this hypothetical cycle, the following metabolic steps might represent significant strategies for both C_3 plants (without temporal separation of reactions) and CAM plants (with day–night separation of reactions) to cope with unfavorable conditions: (1) PEPC can play an important role in the recapture of respired CO_2; (2) malate can be synthesized by OAA reduction via MDH activity, providing a temporary store for CO_2 and reducing equivalents; (3) the reaction catalyzed by ME provides CO_2 and NADPH, which can be utilized in the Calvin cycle, antioxidative system, and amino acid metabolism; and, at last, (4) PEP can be regenerated by PPDK catalysis with ATP consumption (Crecelius et al. 2003; Doubnerová and Ryslavá 2011).

In agreement with the presented panorama, a metabolic link between the CAM cycle and the nitrogen reduction/assimilation was suggested for the atmospheric bromeliad *Tillandsia pohliana* (Nievola et al. 2001; Freschi et al. 2010). This proposition was based on a set of data that indicated a possible connection between the CAM pathway and the nitrate reduction and ammonium assimilation, which were restricted to the dark period in this epiphytic bromeliad. In this sense, the nighttime malate accumulation in *Tillandsia pohliana* could provide carbon skeletons and/or NADH to nitrogen assimilation, while it could be also redirected to act as a counteranion to prevent alkalinization during nitrate reduction (Nievola et al. 2001; Freschi et al. 2010). Moreover, the concomitant nocturnal citrate accumulation suggested the existence of a MDH-linked citrate valve as a possible mechanism for reductant partitioning between the CAM acidification and nitrate reduction during the night (Freschi et al. 2010). Eventually, citrate could also be used as a precursor of 2-oxoglutarate, which, in turn, could be recruited for ammonium assimilation (Martinoia and Rentsch 1994). Therefore, this hypothetical integration between the carbon and nitrogen metabolisms suggested for the CAM bromeliad *Tillandsia pohliana* might be physiologically advantageous for aiding plant survival under severe environment conditions. Further emphasizing the possible link between nighttime citrate accumulation and nocturnal NR activity, it is worth to mention that a typical daytime pattern of NR activity was observed in *Kalanchoë fedtschenkoi*, which is a constitutive CAM plant well known to accumulate malate and virtually no citrate during the night (Chang et al. 1981).

4 Interplay Between Mineral Nutrients and Other Environmental Cues on CAM Expression

Although the essential requirements for plant's photosynthesis are largely satisfied by light, water, and CO_2, plant metabolism also requires a number of mineral nutrients, in particular the macronutrients, which are necessary in comparatively large quantities. Among these elements, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S) are considered the essential

macronutrients required by plants. After uptake from the surrounding environment, mineral nutrients are either immediately assimilated into organic compounds or distributed within the plant tissues for later usage (Amtmann and Blatt 2009; Maathuis 2009). A frequent challenge of studies on nutrients effects in plant metabolism is to separate their role as building blocks of organic matter or cofactors from their potential role as signaling molecules. Besides, it is usually difficult to determine whether the presence/absence of a specific nutrient is directly sensed by the plant or the signal is a derived metabolite (Coruzzi and Bush 2001).

Regardless of these difficulties, it is well established that plant mineral status can markedly influence photosynthesis in various ways. For instance, mineral nutrients are required for chloroplast formation since they contribute to the synthesis of proteins, thylakoid membranes, and chloroplast pigments. In fact the chloroplast is the major source of protein and other nutrients in green plants; thus, the deficiency of mineral nutrients can result in the formation of chloroplasts with lower photosynthetic efficiency. Besides, some mineral nutrients are directly involved in the electron transport chain in the thylakoid membranes, detoxification of oxygen free radicals, photophosphorylation, starch synthesis, and transport of sugars. Additionally, mineral deficiency can also restrain net photosynthesis by affecting the entry of CO_2 through stomata and, ultimately, the CO_2 fixation process (Marschner 1995; Pessarakli 2005).

Even though the mineral nutrition of CAM plants has received relatively scarce attention (Nobel and Berry 1985), some studies have consistently demonstrated that different degrees of nutrient fertilization can influence CAM expression (Ota 1988a, b; Paul and Cockburn 1990; Franco et al. 1991; Ota and Yamamoto 1991; Santos and Salema 1991; Maiquetía et al. 2009; Winter and Holtum 2011). However, most of the data available on this subject indicate an integrated influence of mineral nutrients and other environmental cues modulating CAM photosynthesis. Furthermore, the effects of nutrients on plant photosynthesis are linked to the fact that nutrient concentration in live leaves is largely a function of nutrient availability in the environment. Therefore, all the environmental factors and plant morphological specializations that influence nutrient availability/acquisition can directly impact photosynthesis and the cycling of mineral nutrients in plants (Chapin 1991). This is particularly relevant for plants in extreme habitats with insufficient levels of mineral nutrient resources, such as most CAM plants inhabiting unstable and/or harsher tropical environments, which are described as nutritionally heterogeneous and often specialized (Benzing 1990; Lüttge 2010). Accordingly, a large number of CAM species usually display particular adaptive solutions to improve the acquisition of nutrients which includes both metabolic and morphological adaptations (Benzing 1990; Lüttge 1989, 2010). For example, among epiphytic CAM plants it is relatively common to note the formation of different types of phytotelmata, extensive epiphytic root systems, mycorrhiza association, and myrmecophytism, among other features. Although not CAM specific, CAM plants frequently make use of these morphological/ecophysiological adaptations (Benzing 1990; Lüttge 1989, 2004, 2010).

In view of this information, in terms of ecophysiological plant responses it is also important to consider the nutritional aspect in relation to the action of other environmental factors, especially light and water availability (Lüttge 2004). However, many other environmental cues can also influence the CAM expression. For example, it has been suggested that highly fertilized CAM plants may benefit from elevated atmospheric CO_2 to a greater extent because high nutrient concentrations usually amplify the response of plants to CO_2 enrichment (Poorter and Perez-Soba 2001; Reich et al. 2006; Weiss et al. 2009). Moreover, a multifactor study performed with the CAM species *Kalanchoë pinnata* at high and low irradiance, with and without nitrogen and H_2O deficiency, revealed highly flexible metabolic responses when these different conditions were combined (Lüttge et al. 1991a, b). Similar observations were made by Franco et al. (1991) regarding CAM performance in *Clusia minor* under variable nitrogen supply, light irradiance, and water availability. This same C_3-CAM facultative species also revealed a vast number of flexible photosynthetic responses, which varied from full CAM to CAM idling depending on different irradiances and day: night temperature regime combinations (Haag-Kerwer et al. 1992). The complex interaction of nitrogen supply and light intensity was also studied in some CAM bromeliads such as *Ananas comosus* (Borland and Griffiths 1989), *Neoregelia cruenta* (Fernandes et al. 2002), and *Bromelia humilis* (Fetene et al. 1990). Interestingly, adult plants of *Guzmania monostachia*, a facultative C_3-CAM epiphytic bromeliad (Lüttge 2006), gradually developed low levels of CAM, denoted by increased levels of titratable acidity, when maintained under relatively high irradiance without nutrient fertilization over 3 weeks of treatment (Fig. 1).

Since nutrient and water absorption are closely related processes, some of the effects of water stress on CAM activity in inducible CAM plants might be mediated through effects on nutrients (Paul and Cockburn 1990). The synergistic effect of water and nutritional availability in leaves of the epiphytic bromeliad *Guzmania monostachia* was evident when both water deficit and absence of nutrient fertilization induced an expressive upregulation of CAM expression, which was denoted by increased levels of PEPC activity and nocturnal accumulation of malate (Fig. 2). Moreover, some non-epiphytic species that suffer from intermittent water shortage can also be subject to particular problems of water supply in their habitat which, in turn, implies a certain degree of difficulties for the nutrient acquisition. For instance, Winter and Holtum (2011) studying the drought-induced CAM expression in *Calandrinia polyandra*, an annual succulent herb that grows on sandy or stony nutrient-poor soils, showed a inductive effect of nutrients on CO_2 fixation in the light and a negative effect on dark CO_2 fixation. They also demonstrated for the first time that the drought-induced CAM expression can be reversible in young and mature plants of *Calandrinia polyandra*, which highlighted the role of nutrient supply in the transition from CAM back to C_3. Therefore, this study strengthened the argument that facultative CAM modulation is mainly under environmental control (Winter and Holtum 2011), and mineral nutrients can play an important role in this signaling pathway.

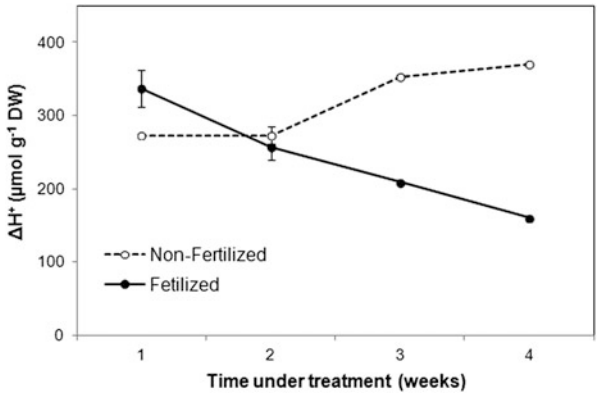

Fig. 1 Effect of nutrient fertilization on nocturnal titratable acidity accumulation (ΔH^+) in leaves of well-watered *Guzmania monostachia* plants for 4 weeks. Adult plants were obtained and acclimatized prior to the treatments as described in Freschi et al. (2010). All plants were watered daily with distilled water and maintained under photosynthetic flux density of approximately 300 μmol photons m^{-2} s^{-1}. One group of plants was supplied once a week with SuperGreen 6:6:6 commercial nutrient solution ("fertilized"), while another group was given only distilled water ("non-fertilized"). Data are mean values ± SE ($n = 6$ plants, 5 leaves from each plant)

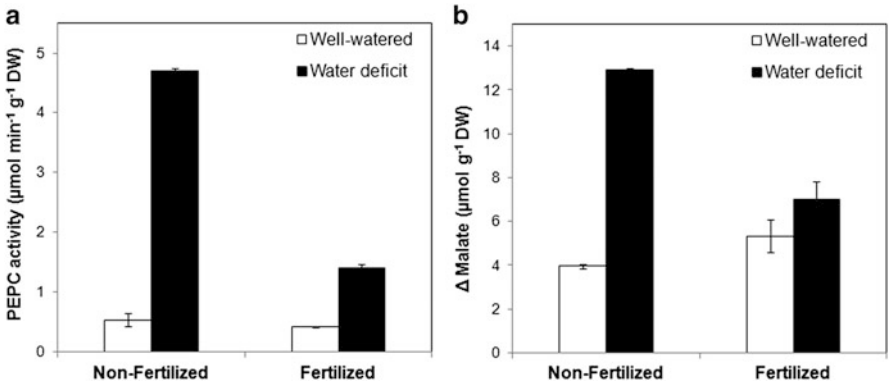

Fig. 2 The interactive effect of fertilization and water supply on the degree of CAM expression in leaves of *Guzmania monostachia*. (**a**) PEPC activity. (**b**) Nocturnal malate accumulation. Adult plants were obtained and acclimatized prior to the treatments as described in Freschi et al. (2010). Detached leaves were maintained for 7 days under photosynthetic flux density of approximately 200 μmol photons m^{-2} s^{-1}. One group of leaves was treated with nutrient Sarruge (1975) solution ("fertilized"), while another group was maintained in distilled water ("non-fertilized"). Water deficit was induced by solution prepared with 30 % of polyethylene glycol 6000 ($\Psi = -1.83$ MPa) and the well-watered treatment received nutrient solutions prepared with distilled water. Data are mean values ± SE ($n = 3$ plants, 5 leaves from each plant)

Among all the essential macronutrients, nitrogen content usually shows the strongest correlation with higher nocturnal acid accumulation in leaf tissues of CAM plants (Nobel 1983; Nobel and Berry 1985). Besides, the absence of nitrogen

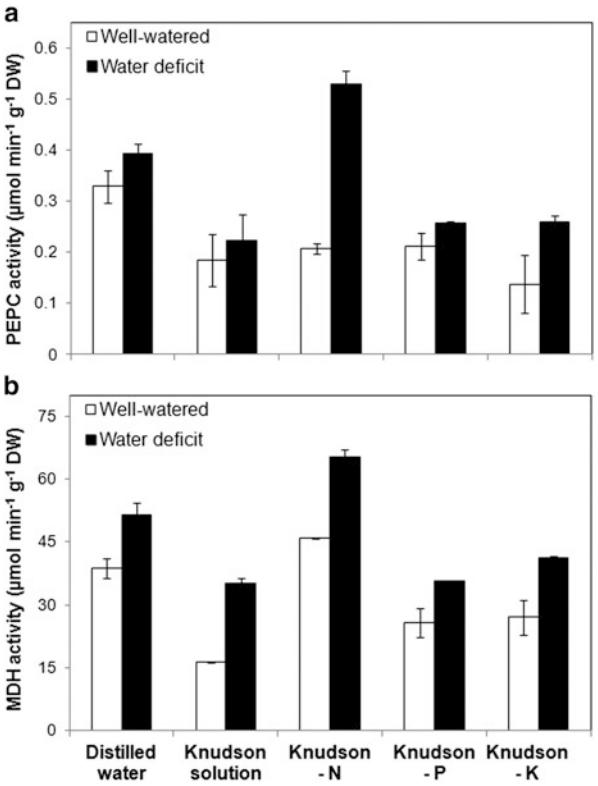

Fig. 3 The interactive effect of nutrients and water supply on the degree of CAM expression in leaves of *Guzmania monostachia*. (**a**) PEPC activity. (**b**) MDH activity. Adult plants were obtained and acclimatized prior to the treatments as described in Freschi et al. (2010). Detached leaves were maintained for 7 days under photosynthetic flux density of approximately 200 µmol photons m^{-2} s^{-1}. One group of leaves was treated with nutrient Knudson (1946) solution ("fertilized"), while another group was maintained in distilled water ("non-fertilized"). Water deficit was induced by solution prepared with 30 % of polyethylene glycol 6000 ($\Psi = -1.83$ MPa) and the well-watered treatment received nutrient solutions prepared with distilled water. Data are mean values ± SE ($n = 3$ plants, 5 leaves from each plant)

sources in the nutritional solution fed to the *Guzmania monostachia* under water deficit induced the most expressive upregulation of CAM expression among all macronutrients tested, which was indicated by increased levels of both PEPC and MDH activities (Fig. 3). In agreement with these results, Ota (1988b) observed that CAM expression was stimulated when the nitrogen sources were removed from the nutrient solution where *Kalanchoë blossfeldiana* plants were cultivated. Besides, nitrogen and phosphorus deficiencies are promoters of CAM expression in drought-exposed seedlings of *Clusia minor*. However, it is suggested that the stimulatory effects of phosphorus deficiency on CAM expression occur via phosphate-deficient-induced nitrogen deficit (Maiquetía et al. 2009). In fact, nitrate deficiency

is also known to result from phosphate shortage. Hence, nitrate may be deficient not only when it is withdrawn from the growing medium, but also in conditions of low water potential and phosphate deficiency (Schjorring 1986; Paul and Cockburn 1990).

5 CAM Modulation by Different Levels and Sources of Nitrogen

Nitrogen is quantitatively the mineral element that plants require in the largest amounts and is the most important nutrient in the plant body. Therefore, nitrogen is a limiting factor in plant growth and development (Hu and Schmidhalter 2005; Kraiser et al. 2011). This macronutrient is taken up from the environment and is used for several metabolic purposes, such as the synthesis of many plant cell components, including nucleic acids, proteins, and cofactors, as well as signaling, storage, and defense molecules. Besides, leaf levels of nitrogen are often correlated with photosynthetic capacity (Gessler et al. 2008). Chloroplastic proteins represent about 75–80 % of the total stored nitrogen in green leaf cells, mainly represented by enzyme proteins. In C_3 leaves, RUBISCO accounts for up to 50 % of the stored nitrogen, while this enzyme corresponds only to approximately 20 % of stored nitrogen in C_4 leaves (Marschner 1995; Good and Beatty 2011; Kant et al. 2011; McAllister et al. 2012). In addition, it has been suggested that CAM plants, as proposed for the C_4 pathway, would also need less RUBISCO than C_3 leaves due to their CO_2 concentrating mechanism which could suppress photorespiration, hence requiring reduced nitrogen investment in RUBISCO protein for CO_2 fixation (Raven et al. 1988; Griffiths 1989; Robe and Griffiths 1994; Raven and Spicer 1996).

Plant nitrogen use efficiency (NUE) is usually defined as the total biomass yield produced per unit of applied nitrogen. However, the definition of NUE is inherently complex since it is a function of multiple interacting genetic and environmental factors that involves nitrogen uptake, assimilation, and translocation/remobilization (Hu and Schmidhalter 2005; Masclaux-Daubresse et al. 2010; Xu et al. 2011; McAllister et al. 2012). It is well established that NUE is usually higher in C_4 than C_3 plants, since much higher rates of CO_2 fixation are achieved with lower leaf nitrogen content (Monson 1989; Marschner 1995). This is supposed to be the result of the CO_2 concentrating mechanism in C_4 plants leading to CO_2 saturation in the site of RUBISCO activity. Consequently, C_4 plants require significantly less RUBISCO enzyme than C_3 plants in order to sustain high rates of photosynthesis (Björkman et al. 1976; Brown 1978; Sage and Pearcy 1987).

A similar NUE tendency observed in C_4 has been predicted for CAM plants (Osmond et al. 1982; Santos and Salema 1991); however, the efficiency of CAM species to use nitrogen seems to be highly species specific and variable according to both ontogenetic and environmental conditions (Lüttge 2004). In fact, *Kalanchoë*

daigremontiana and *Kalanchoë tubiflora* showed NUE values lower than expected for CAM plants (Widmann et al. 1990) and the NUE of some other CAM plants was low because of the reduced rates of photosynthesis in vivo (Robe and Griffiths 1994). Additionally, the NUE of some C_3 species was higher than that of CAM species (Widmann et al. 1993) and relatively elevated NUE values found in some CAM species not necessarily coupled with high productivity (Widmann et al. 1993). Altogether, the literature information currently available does not support particularly high NUE in CAM plants (Widmann et al. 1993; Lüttge 2004). Therefore, the adaptive success of most CAM species under low nitrogen supply seems not to be dependent on making the most effective use of nitrogen to maximize CO_2 assimilation to produce biomass. Instead, these plants attempt to increase their chance of survival under harsh environments by changing from biomass production towards provisions for life preservation (Widmann et al. 1990, 1993; Robe and Griffiths 1994).

Furthermore, nitrogen nutrition has been reported as an additional environmental cue that influences CAM pathway since both nitrogen-deficient treatments and the supply of different nitrogen sources can affect the level of CAM expression (Ota 1988a, b; Ota et al. 1988; Borland and Griffiths 1989; Paul and Cockburn 1990; Ota and Yamamoto 1991; Santos and Salema 1991). However, contrasting observations have been reported regarding the photosynthetic responses of CAM plants to nitrogen supply and tissue nitrogen content. For example, some CAM species displayed increased CAM activity in response to increased nitrogen supply and/or tissue nitrogen content (Nobel 1983; Winter et al. 1982; Borland and Griffiths 1989; Franco et al. 1991). Conversely, other CAM plants showed increased CAM expression upon nitrogen deficiency (Ota 1988b; Paul and Cockburn 1990; Santos and Salema 1991). Accordingly, nitrogen deficiency increased CAM performance in *Kalanchoë blossfeldiana* by causing higher rates of nocturnal CO_2 fixation, ΔH^+, and PEPC activity (Ota 1988a, b). Santos and Salema (1991, 1992) verified that CAM expression in *Kalanchoë lateritia* was increased to a greater extent at intermediate nitrogen supply, indicating that CAM photosynthesis can be also affected by a certain range of nitrogen concentration and that nitrogen levels above or below a specific optimum will reduce CAM activity.

A further degree of complexity is added in this scenario when the involvement of specific nitrogen ions is considered as signaling molecules in CAM modulation. For example, CAM activity in *Mesembryanthemum crystallinum* was enhanced by nitrate removal from the nutritional solution prior to salt treatment (Paul and Cockburn 1990). Besides, nitrate as the sole nitrogen source induced a more pronounced CAM activity in *Kalanchoë blossfeldiana* than ammonium supply, suggesting that ammonium could depress PEPC activity and CAM pathway in this species (Ota 1988a; Ota et al. 1988; Ota and Yamamoto 1991). Another evidence that supports the idea that the nitrogen source might affect CAM photosynthesis in a specific manner is given by the distinct degree of CAM expression, denoted by PEPC and MDH activities, observed in drought-exposed *Guzmania monostachia* leaves under treatments with nitrate, ammonium, or urea. Accordingly, in this C_3-CAM facultative epiphyte bromeliad the presence of ammonium as

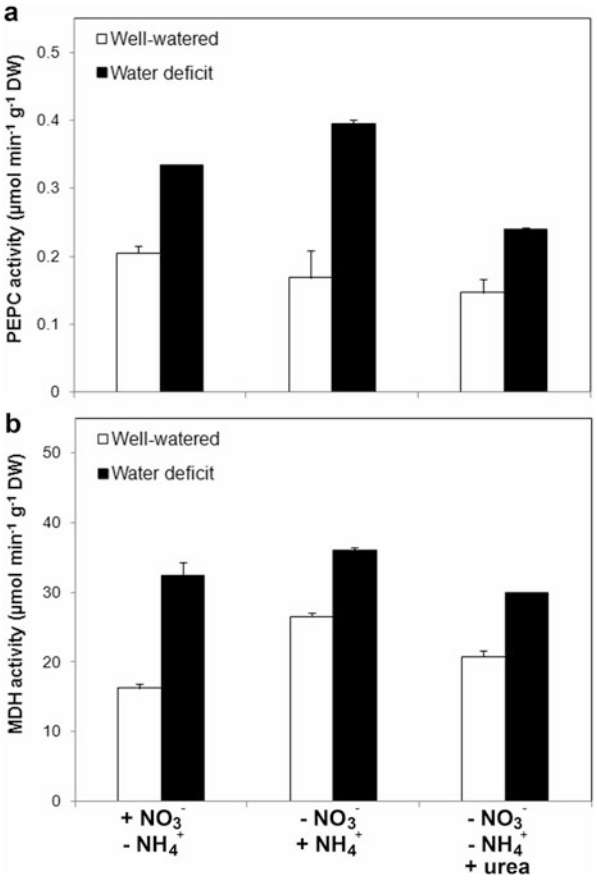

Fig. 4 The interactive effect of water supply and different nitrogen sources (NO_3^-, NH_4^+, or urea) on the degree of CAM expression in leaves of *Guzmania monostachia*. (**a**) PEPC activity. (**b**) MDH activity. Adult plants were obtained and acclimatized prior to the treatments as described in Freschi et al. (2010). Detached leaves were maintained for 7 days under photosynthetic flux density of approximately 200 µmol photons m^{-2} s^{-1}. One group of leaves was treated with water deficit induced by nutrient solution (based on Knudson formulation, 1946) prepared with 30 % of polyethylene glycol 6000 ($\Psi = -1.83$ MPa) and the well-watered-treated leaves received nutrient solutions prepared with distilled water. The nitrogen sources were supplied at equivalent concentrations. Data are mean values ± SE ($n = 3$ plants, 5 leaves from each plant)

the sole nitrogen source seemed to be slightly more inductive for CAM operation than the other sources tested (Fig. 4). Interestingly, studies with the CAM plants *Clusia* and the tank bromeliad *Neoregelia cruenta* reported a strong preference for ammonium over nitrate (Arndt et al. 2002; Fernandes et al. 2002). Altogether, this evidence indicates the importance of specific effects of nitrate and ammonium ions in the CAM modulation rather than an influence of nitrogen status itself. In fact, it is well known that plants have uptake systems for both nitrate and ammonium with different affinities which aid plants to deal with the concentration heterogeneity and dynamic variations of these nitrogen ions in the environment (Xu et al. 2011). Nonetheless, future research is required to unravel the effects and the respective physiological mechanisms of the nitrogen source on CAM photosynthesis.

6 Conclusions and Perspectives

CAM plants usually inhabit unstable and/or stressful environments where a variety of external cues, such as water and nutrient availability, can impact the plant metabolism depending on the genotype and growing conditions. Mineral nutrients are essential compounds that can influence CAM expression and some evidence also indicates that CAM operation can affect the nitrogen metabolism to some extent. Among all essential nutrients for plants, nitrogen has been shown as the mineral macronutrient more closely related to CAM modulation, whereas scarce information regarding the effects of other nutrients on this photosynthetic pathway is currently available. However, even nitrogen nutrition has received much less attention in CAM plants than in either C_3 or C_4 species. Furthermore, different sources of mineral nitrogen, such as nitrate and ammonium, can regulate CAM expression in specific ways, indicating that particular nitrogen ions might be more important for signaling CAM adjustments than the nitrogen status itself.

The challenge now is to unravel how mineral nutrients and other environmental signaling pathways are connected to the core CAM machinery and to establish how these signaling mechanisms influence the metabolic fitness under various environmental constraints. Further challenges in this field include (1) defining the tolerance limits of nutrient deficiency for CAM plants; (2) determining the impact of treatments with the absence or reduced levels of more than one type of mineral nutrient at the same time; (3) characterizing the impact of simultaneous treatments of nutrient scarcity with other environmental stresses; (4) distinguishing the effects of mineral nutrient and water availability on CAM expression—one possible strategy to deal with this challenge could be the use of hyperosmotic nutritional solutions, such as PEG solution ; and (5) investigating the mechanisms and molecules that participate in the signaling pathway transducing nutrient absorption/perception into CAM modulation. Certainly plant models showing increased plasticity to switch between C_3-CAM could provide excellent experimental material for studies regarding these topics.

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Guttation: Quantification, Microbiology and Implications for Phytopathology

Sanjay Singh

Contents

Abstract Guttation is the process of liquid exudation from hydathodes situated on the tip, along the margins and adaxial and abaxial surfaces of leaves. Hydathodes, also known as water stomata or water pores, unlike stomata, are always open representing the path of least resistance to the liquid outflow from them. Guttation fluids contain a variety of living and non-living ingredients. The living materials include algae, fungi, bacteria, viroids and viruses. The non-living organic constituents include toxins, mycotoxins, alkaloids, proteins, enzymes, sugars, amino acids, volatiles, hormones, vitamins, etc., and the inorganic components include Na, K, Ca, Mg, Mn, B, Co, Zn,

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Se, Ni, Fl, Si, As, Al, Cl, NH_4, NO_3, PO_4, SO_4, CO_3, HCO_3, etc. This review highlights various techniques for measuring guttation, both qualitative and quantitative, and their use and utility are discussed. Further, the microbiological aspects of guttation, with particular reference to the incidence of algal, fungal, bacterial and viral diseases and toxins produced by these pathogenic organisms, are described. The production of new chemicals by host plant as strategies to protect from harmful effects of pathogens is also outlined. The goal here is to stimulate discussion on our gaps of knowledge in the physiology and biochemistry related to guttation including genetic aspects, and the microbiology associated with guttation. A long-range goal is to design and create improved plant types with increased productivity, and developing effective control measures for plant diseases, to help sustain agriculture in a world with a burgeoning human population. A better understanding of the physiology behind guttation might contribute substantially to this aspiration.

1 Introduction

The topic of guttation was last briefly reviewed 56 years ago by Stocking (1956) followed by slightly upgraded intervening descriptions by Logvenkov (1993a, b). Traditionally, guttation was considered as a means to maintain xylem flow when plant transpiration becomes restricted in moist atmosphere and is often observed during early morning or late hours of the day. Since publication of the Stocking review, however, substantial work has modified and extended the facts and hypotheses related to guttation (Pedersen et al. 1997; Komarnytsky et al. 2000; Grunwald et al. 2003; Pilot et al. 2004; Chen and Chen 2005; Pillitteri et al. 2008; Rybicki 2009; Peterson et al. 2010; Wang et al. 2011; Sharabani et al. 2012; Singh and Singh 2013). The current review highlights the techniques for the quantification of guttation, and microbiological aspects including the implications for phytopathology as various microorganisms such as algae, fungi, bacteria, viruses, etc. are often detected in guttation fluid. The question of the role of guttation in pathogenicity and parasitism of plants is particularly interesting and important. Most guttates contain substances that promote the growth of many facultative parasites and saprophytes. The pH of the exudate is also favourable to the growth of microorganisms. Aside from the common salts and sugars, the guttated fluid also contains organic solutes, enzymes, vitamins, reducing and oxidising agents (Goatley and Lewis 1966; Gay and Tuzun 2000; Grunwald et al. 2003; Pilot et al. 2004) and specific growth-promoting substances such as auxins, gibberellins, cytokinins, ABA, etc. as well (Aloni et al. 2005; Fletcher and Mader 2007), not yet fully investigated which may be essential in the initiation and development of some specific pathological processes. The frequent occurrence of the guttate in the form of films and minute drops on the laminal surfaces of leaves offers a favourable starting point for infection.

Considering that the guttation fluid is exuded from hydathodes and hydathodes that are always open, then simple reasoning would suggest that plants must have mechanisms to defend themselves from invasion by algae, fungi, bacteria and

viruses. At least part of the answer must be in the composition of guttation fluid. It is, however, astonishing that after 45+ years of research into the composition of guttation fluid (Goatley and Lewis 1966), it is sometimes understood that the only solutes present in guttation fluid are glutamic acid and glutamine. However, recent information suggests that guttation fluid, among other chemical factors, contains small anti-pathogenic peptides (APPs) that serve to restrict or inhibit microbial proliferation (Grunwald et al. 2003). To control aerial disease onset it would make sense to spray on, or genetically transform the plant to increase the production and/or secretion of these peptides to kill pathogens. Such a strategy represents yet another way to control aerial infestations of plants by microorganisms to improve productivity, but it has yet to be developed or tested. As early as 1936, Johnson in his studies on the relation of root pressure to plant diseases stated that conditions favourable to guttation often result in water soaking of the leaves, facilitating infection by pathogenic microorganisms which otherwise would have difficulty in gaining entrance. Besides providing a liquid vehicle for bacterial and other pathogens to enter the host plant, the water of guttation causes an increase of relative humidity under and around the plant parts in a manner similar to the action of dews and other kinds of precipitation. Guttation, therefore, should be considered in relation to disease epidemiology in dry climates, and in areas where irrigation is practised. Although guttation and dew precipitation are frequently confused, the two phenomena are separate and causatively and otherwise distinct and do not necessarily occur at the same time.

2 Structure of Hydathodes

Transpiration is the loss of water as vapour from stomata of leaves, but guttation is exudation of liquid water via special structures called ‘hydathodes’ and sometimes also known as ‘water stomata’ or ‘water pores’ (Figs. 1 and 2). They are located at the tips, along the margins and adaxial and abaxial surfaces of leaves and found in a wide range of plant species (Dieffenbach et al. 1980a, b; Lersten and Curtis 1982, 1985, 1991; Sperry 1983; Maeda and Maeda 1987, 1988; Chen and Chen 2005, 2006, 2007; Singh et al. 2009). Morphologically, they form natural openings, and unlike stomata they are permanently open representing a pathway of least resistance to the flow of fluid out of leaf. The distribution of these structures on the leaf surfaces is variable. They can occur singly with fairly regular spacing, but often they are found in clusters of one to several at each site.

The anatomy of the hydathode reveals that each hydathode is formed by translucent cells and outwardly they appear as stomata-like pores in the epidermis or epithem connected to tracheary endings having large chamber with masses of thin-walled parenchymatous and loose tissue surrounded by sheath layer. It is the parenchymatous loose tissue lying beneath the hydathode, which is known as ‘epithem’ or ‘transfer tissue’, that is involved in absorption and secretion. As regard to their function, there are two types of hydathodes, viz. epidermal hydathodes and epithemal hydathodes

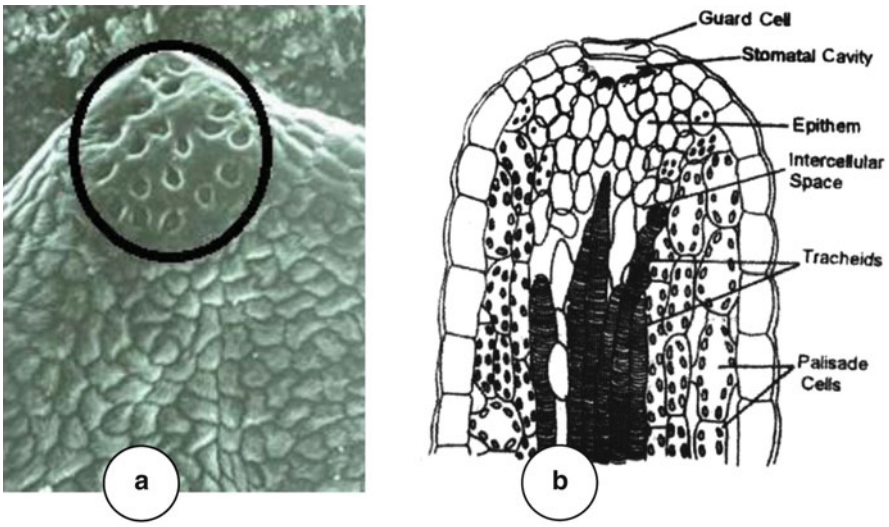

Fig. 1 External and internal features of hydathodes in *Ficus formosana* Maxim. f. *Shimadai* Hayata. (**a**) Magnification of hydathodes found in group (*encircled*). (**b**) Drawing of a longitudinal section of a hydathode (*Source*: Chen and Chen 2005)

exuding actively or passively, respectively. Ultrastructurally, the epithem cell has a dense cytoplasm, numerous mitochondria, an extended endoplasmic reticulum, many small Golgi-derived vesicles and proliferate peroxisomes with abundant plasmodesmata interconnecting the cells. Plasmalemmasomes are also found on the plasma membrane of the epithem cells.

3 Measurement of Guttation

There have been numerous investigations in recent years on guttation, but unless stringent precautions are taken it is difficult to recover adequate volumes of material for experimentation without the accidental introduction of contaminants. However, its quantification and determination of composition are essential for microbiological, phytopathological, ecological, physiological, biochemical, pharmaceutical and agricultural research, now especially in an era of biotechnology and nanotechnology. Pedersen in Denmark (Pedersen 1993, 1994), Raskin at Rutgers in New Jersey (Komarnytsky et al. 2000), Wagner at the University of Kentucky (Wagner et al. 2004) and Singh in India (Singh et al. 2008, 2009) have been successful in developing methods for the collection of fluid and making a variety of measurements. Qualitative assessments have been made either by simple visual

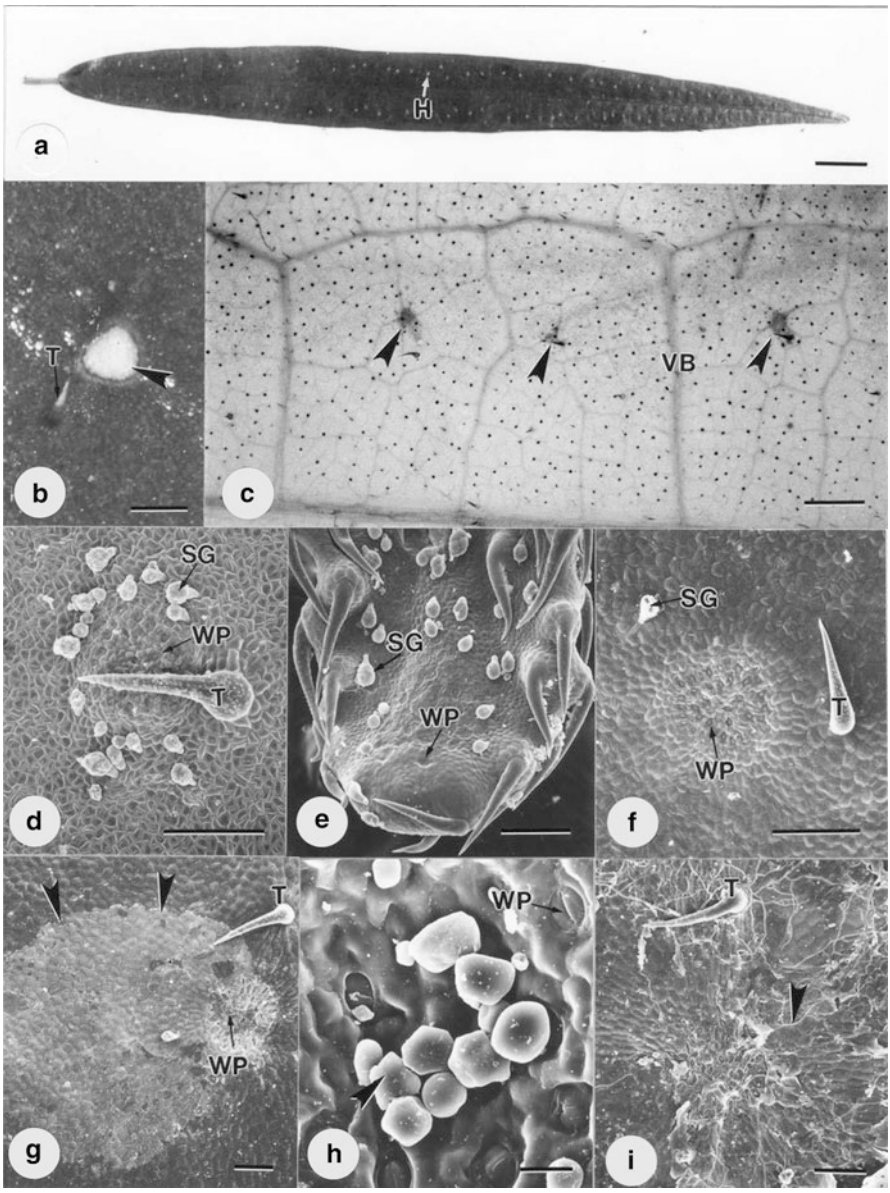

Fig. 2 External features of the laminar hydathodes in *Ficus formosana* Maxim. f. *Shimadai* Hayata. (**a**) Hydathodes on the adaxial surface of leaf and scattered in a linear arrangement between mid-rib and leaf margin. *White points* indicate hydathodes. (**b**) Magnification of a hydathode. Hydathode (*arrowhead*) consists of a trichome and a group of water pores on leaf upper epidermis. (**c**) Leaf showing the venation pattern and three hydathodes, which is associated with vein-end junctions of the venation (*arrowheads*). (**d**) SEM micrograph of a young leaf showing the laminar hydathode consisting of a giant trichome, numerous salt-glandular trichomes and a group of water pores. (**e**) SEM micrograph showing a hydathode at leaf tip of a young leaf. (**f**) SEM micrograph showing a mature laminar hydathode with a trichome, a remnant salt-glandular trichome and a group of water pores. (**g**) SEM micrograph of a hydathode with chalk scales (*arrowheads*) dispersed on outer

imaging and assessment or by using electronic wetness sensing detectors (Richards 2004). Quantitative assessment, on the other hand, involves the collection of fluid in microglass capillaries, adsorption of exudates on adsorbent material such as blotting paper or collection of material in test tubes or petri dishes as the fluid drips from leaves. A brief description of these techniques is given hereunder.

3.1 Qualitative Assessment

Leaf wetting by guttation has important phytopathological implications (Sect. 4). To assess it in comparison to dew position and evaporation it is necessary to study the shape and size of guttation droplets.

3.1.1 Image Analysis of Guttation Droplets

Richards (2004) examined droplets of guttation exudate plants and recorded their images. The shape of the droplets and its location on the convex versus concave sides of the plant coleoptiles were quantified by a model dominated only by surface tension parameters.

3.1.2 Measurement of Shape of Guttation Droplets

In the morning on a sunny day one can sometimes see glare spots associated with uncoloured 'rainbow' caustics due to the sunlight reflected from the surface of dew or guttation drops. Lock et al. (2008) and Adler et al. (2008) have shown that these dewdrop reflection rainbows were due to places on the droplet (i.e. from an 'inflection circle') where the Gaussian curvature becomes zero. Though this technique can be used for measuring the shape of guttation droplets, such measurements are not quantitative by any means or standard.

Fig. 2 (continued) surface of leaf after the guttate fluid evaporated. (**h**) SEM micrograph showing salt crystals (*arrowhead*) precipitated on outer surface of the water pores. (**i**) SEM micrograph showing fungal mycelia (*arrowhead*) growing on the outer surface of a hydathode. Some of them are even intruding into the inner parts of the hydathode through the water pore. (Bar scales: (**a**) = 1 cm; (**b**) = 0.3 mm; (**c**) = 1 mm; (**d–g**) and (**i**) = 100 μm; (**h**) = 10 μm). (*Source*: Chen and Chen 2005). *H* hydathodes, *SG* salt-glandular trichome, *T* trichome, *VB* vascular bundle, *WP* water pore

3.1.3 Measurement of Size of Guttation Droplets

The size of guttation droplets found on leaf blade tips also provides an estimate of guttation which might be important from ecological point of view aiding to plant survival under semi-arid conditions. Hughes and Brimblecombe (1994) found an average guttation droplet diameter of 1.49 ± 0.16 mm, compared with 0.20 ± 0.02 mm for true dew droplets on the grass *Holcus lanatus* L. (Yorkshire fog) at a site in rural Norfolk, UK. The average total volume of guttation water exuded per grass blade per night was $1.0 \pm 0.3 \times 10^{-7}$ dm^3, which represents about 0.1 mm of precipitation; guttation supplied about the same amount of water as dewfall to a short grass surface. The authors suggested that about 8 % of the mean daily June–August net radiation in southern England would be needed to evaporate the average dew and guttation-derived leaf wetness, which totalled 0.25 ± 0.04 mm signifying the ecological significance of dew and guttation.

3.2 Quantitative Measurement

Quantitative measurements are essential for understanding the physiological functions, the ecological relevance and the phytopathological implications of guttation. Only quantitative measurements allow obtaining the important information of concentration and total amounts of solutes in the exudates. Therefore, below are briefly described various techniques for quantitative measurements of guttation content.

3.2.1 Mass Collection of Guttation Fluid from Leaf Drippings

Raleigh (1946), Smith and Olien (1978) and Luo and Goudriaan (2000) collected guttation water, either in test tubes or on circular blotting papers of 9 cm diameter each installed inside the canopy, as it dripped from the leaf tips. The collected material was used for the study of the effects of ions on the intensity of guttation in tomato, pathogenicity in barley, or comparison of guttation with the amount of dewfall on rice crop, respectively. However, it is difficult to find a satisfactory index for the amount of guttation collected and used in these studies as there are uncertainties about the true volume of collected material. These methods of collection should not be regarded as precise because of incomplete recovery of guttation fluid as only drops beyond a certain size will detach and fall, and loss of water through evaporation during handling will change the concentration of dissolved solutes. Further, it is impossible to distinguish between fluid from leaf tips, edges and surfaces, and contamination with dew is yet another confound.

3.2.2 Pedersen's Technique for Measuring Guttation

Guttation may be used as an effective and useful tool for studying transport of water, nutrients, hormones, etc. in non-transpiring plants such as those of submerged aquatic plants. While studying the long-distance water and nutrient transport, Pedersen (1993) devised a special technique of inducing and collecting droplets oozing from tips of leaves of aquatic plants *Lobelia dortmanna* and *Sparganium emersum*. With the leaf tips held in a small, enclosed and highly humid atmosphere, guttation drops emerge which could be collected with precision by means of pre-weighed microglass capillaries. The increase in weight after fluid collection provided a relatively precise quantitative measurement of the amount of fluid collected.

3.2.3 Raskin's Technique for Measuring Guttation

The technique developed by Raskin and colleagues involves collection of guttation fluid by means of a handheld pipette or vacuum suction into an aspirator bottle (Komarnytsky et al. 2000). They used this technique of collection of guttation fluid for recombinant protein production which can be performed throughout a plant's life, thus providing a continuous and non-destructive system. Though this technique is quite useful but might prove time-taking for large scale screening for guttation efficiency at field level in a breeding programme of field crops or horticultural crops.

3.2.4 Wagner's Method of Measuring Exudates

Wagner et al. (2004) developed a technique that involves touching individual exudate droplets atop glandular secreting trichomes (GSTs) with a micro-capillary containing a solvent that readily dissolves exudate. Prior to exudate removal, rapid rinsing with methylene chloride or acetonitrile of leaf surfaces is preferable which does not appear to leach components from within the leaf. GSTs being surface protuberances, their exudates are more easily accessed by mechanical means than cells or components of cells embedded in tissues within the plant. Although tedious, this method has the advantage of making contact only around glands, and therefore yields potentially pure exudate. This approach can quickly provide complete removal of large quantities of exudate and allows for accurate estimation of the exudate amount on a surface area basis. However, being relatively tedious this procedure inherits the weaknesses of assessing non-trichome cuticular components of guttation fluids as well. Though this technique is good for collecting chemicals present in GSTs but cannot be used as such in a crop breeding programme under field conditions.

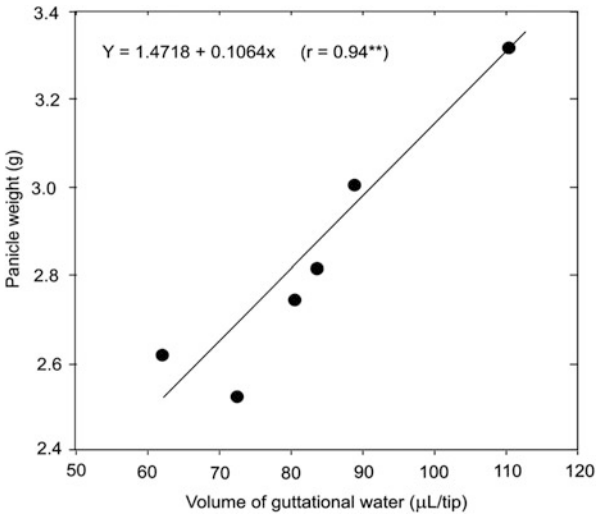

Fig. 3 The relationship for six rice cultivars between the rate of guttation during pre-heading stage and their panicle weights (the yield sink potential) (*Source*: Singh et al. 2008)

3.2.5 Singh's Blotting Paper Technique for Measuring Guttation

For physiology, ecology, molecular biology, phytopathology and relevant breeding activities it is indispensable to have a method suitable for large-scale screening of amounts of guttation fluid exuded and quantification of its solute contents. While studying the physiology of guttation in rice, Singh et al. (2008, 2009) developed such a technique for quantitative measurement of guttation that is easy, simple, accurate, non-invasive and quick to perform. Since no added chemical is used, it is cost-effective and environmental-friendly too. Further, this technique does not need costly and cumbersome equipment either and requires much less time than other known laboratory or field methods and techniques for guttation measurement. The technique involves collection by adsorption of exuded droplets from the tips, edges or surfaces of leaves, as one may desire, on blotting paper pieces of convenient size held by forceps, before they trickle down from the exuding sites and placing them immediately into small and airtight weighing glass vials. The amount of fluid exuded is determined gravimetrically by drying the filter paper pieces soaked with guttation fluid in the oven at 80 °C for 24 h. The glass vials are then stoppered and weighed and the amount of guttation fluid can be expressed either on individual leaf or area or weight basis per unit time. However, for the sake of simplicity it can also be expressed volumetrically, ignoring the solute contents present therein which are insignificant by weight. This technique is novel and holds out good promise for selecting cultivars exhibiting high rates of guttation from a large plant population and breeding guttation-efficient crop varieties transgenically for yield improvement as a positive correlation between the intensity of guttation and the yield of panicles (sink potential of panicles) in rice varieties has been demonstrated (Fig. 3) (Singh et al. 2008, 2009). Further, the chemical identification and measurements of organic and inorganic constituents by eluting them with suitable solvents may

also be performed. However, prior to exudate collection, rapid rinsing with methyl or ethyl alcohol of blotting papers is preferable for sterilising and freeing them from possible contaminants.

4 Microbiology of Guttation

4.1 Mode of Entry of Pathogens in Host Tissues

Plant pathogens include algae (occasionally), fungi, bacteria, viroids, viruses, fastidious bacteria, etc. Numerous factors affect the ability of a pathogen to infect a plant tissue, including plant age, tissue type, environmental factors, pathogen type, inoculum levels and methods of pathogen dissemination. Pathogens can gain entry into plants via stomata, hydathodes, wounds, lenticels, broken trichomes, root hairs and lateral root outgrowths.

4.2 Growth Requirements of Plant Pathogens in Host Tissues

Fungi can establish themselves on the surface of healthy tissues, enter passively natural openings or wounded tissues, enter actively as a result of the production of cuticle- and cell wall-degrading enzymes or gain entry through the penetration of tissues by structures called pegs that develop from the base of the appressorium. Some fungi form haustorial connections with host cells through which they then extract nutrients from the host by establishing a strong metabolic sink. Generally, pathogens must establish infection sites intercellularly where suitable moisture, nutrients, temperature and osmotic relations will more readily support growth. Viruses, fastidious bacteria and mollicutes are all obligate, systemic parasites and enter plants via insect vectors or through wounds.

4.3 Salt Incrustation by Guttation and Pathogen Infection

The guttation drops on a plant have three possible fates. They may roll off or be blown off the leaf and enter the soil, be evaporated or be sucked into the leaf, as most frequently happens on undisturbed plants (Curtis 1943). Numerous observations have been made on several plant species that the guttation drops are, in most instances, sucked back into the leaf. This sucking of guttation drops back into the vascular system enhances the chances of entry of pathogens causing infections. On the other hand, when guttate solution evaporates in the daylight, salt incrustation can be precipitated around the vicinity of the surface of hydathodes (Figs. 1 and 2). Because

guttate fluid is rich in nutrients for microbial growth, several bacterial cells and fungal hyphae growing near hydathodes have often been noticed. Hydathodes have been found to serve as an extremely efficient infection court for the bacterial pathogens (Carlton et al. 1998; Fukui et al. 1999) and as a practical system for studying early infection events in bacterial pathogenesis (Hugouvieux et al. 1998). Thus, guttation fluid is an ideal medium for the growth of certain microorganisms and disease infection (Ivanoff 1963). In addition, epithem cells have dense cytoplasm, numerous mitochondria, extended endoplasmic reticulum systems and considerable numbers of small vesicles within the cytoplasm. The epithem cells could modify the content of guttate fluid along the transport route and indirectly affect the salt incrustation (Dieffenbach et al. 1980b). The plasmodesmata connected with other cell components seem to increase the transport efficiency between different epithem cells and cells of the sheath layer (Cantrill et al. 1999; Crawford and Zambryski 1999). Microscopy has confirmed that the hydathodal area, which is an apical, marginal and laminar opening (Chen and Chen 2005), undergoes structural changes with leaf age; a matrix of microorganisms develops in the leaves and probably restricts water flow by clogging the hydathodes (Takeda and Glenn 1989; Pedersen et al. 1997).

4.4 Chemicals of Guttation and Pathogenic Abnormalities

Guttation fluid is considered to be an ideal medium for fungal, bacterial and viral growth. It constitutes the habitats that appear to be favourable for pathogens' development (Bald 1952). Besides providing a liquid vehicle for bacterial and other pathogens to enter the host plant, the water of guttation causes an increase of relative humidity under and around the plant parts in a manner similar to the action of dews and other kinds of precipitation. Examination of guttation fluid may also indicate the pathogenic status of fruits and developing grains. As stated earlier, guttation, therefore, should be considered and taken into account in explaining disease epidemiology in dry climates and in areas where irrigation is practised. It is, therefore, likely that sometimes in certain cases guttation may cause disease infections by releasing toxins in the host plants. However, the early views and belief about injurious effects of guttation water have now been re-evaluated in the light of recent findings and new discoveries relating to its structural biology (Chen and Chen 2005), hydathodal initiation and development (Pillitteri et al. 2008; Peterson et al. 2010; Wang et al. 2011), chemistry and pharmaceuticals (Goatley and Lewis 1966; Komarnytsky et al. 2000; Rybicki 2009), microbiology (Kerstetter et al. 1998; Gay and Tuzun 2000; Pilot et al. 2004; Shepherd and Wagner 2007; Slewinski et al. 2009; Schoelz et al. 2011; Koulman et al. 2012; Ryan et al. 2011) and agricultural implications (Singh et al. 2008, 2009).

4.5 Pathogens in Guttation Fluid

The plant pathogens such as algae, fungi, bacteria and viruses with their ability to infect plant tissues have been detected in guttation fluids obtained from a number of plant species including field crops, horticultural crops, vegetable crops and tree crops, etc. Below is described briefly their phytopathological impact in host plants:

4.5.1 Phycology of Guttation

Cyanobacteria, commonly referred to as algae, are found to have migrated to the leaf tip where guttation fluid is present and some of them such as *Oscillatoria* spp. are thought to be associated with yellow spot disease. Another group of algae, i.e. *Vaucheria* which guttates and belongs to genus of Xanthophyceae or yellow-green algae, is one of only two genera in the family Vaucheriaceae. *Vaucheria* exhibits apical growth from the tip of filaments forming mats in either terrestrial or freshwater environments. An analysis was made of the guttation fluid secreted by *Vaucheria*, and K, Ca, Mg, carbonates, phosphates and sugar were present in the fluid with dry matter varying between 0.5 and 0.6 % (Guiry and Guiry 2008; Lee 2008). Apart from higher plants, biological secretions have also been noted in algae, and interestingly, to human advantage genetically modified eco-friendly algae have been produced (Daniell et al. 2002). However, very little work has been done on the phycology of guttation and/or guttation by algae themselves; hence, more rigorous work is required on natural and genetically modified algae.

4.5.2 Mycology of Guttation

A number of studies have been conducted on the correlation between guttation and fungus infection (Yarwood 1952). A fungal disease in plants is usually caused by only one species; however, a fungal species can attack one or several plant species. Still, however, completely successful colonisation occurs only in limited cases. Plants have no immune system, though co-evolution of plants and pathogens has created a multifaceted relationship resulting from the exchange of molecular information between the species. Based on this, plants have developed a complex surveillance system with an array of defence mechanisms. For example, when young detached bean leaves were floated with their adaxial or abaxial surfaces on water in a humid chamber, water of guttation usually formed on the opposite leaf surface exposed to the air. When bean leaves were inoculated with dry spores of *Uromyces* or *Colletotrichum lindemuthianum*, the resulting infection was positively correlated with the amount of guttation. Lewis (1962) studied the susceptibility of three cereals to *Claviceps purpurea* using their guttation fluids as media for the growth of germinating spores of this fungus. Rye guttation fluid produced the most growth and barley fluid the least. The degree of susceptibility was correlated with

the amount of growth of the parasite in vitro. Therefore, it is evident that fungi would grow abundantly in guttation drops, but their effects on plants would depend upon several internal and external factors (Endo 1967). It is interesting to know that guttation is also a common phenomenon of fungal mycelia. However, little is known about the composition of such exudates. In fact, the chemical constituents of plant guttation represent the combined secretions of both plant and pathogenic organisms (Fig. 4). For example, in droplets of *Penicillium* species, penicillin was found in approximately the same concentration as in the underlying culture. Gareis and Gareis (2007) found high levels of ochratoxins in guttation droplets from *Penicillium* species. Grovel et al. (2003) reported the excretion of gliotoxin in an exudate of *Aspergillus fumigatus*. Droplets of *Sclerotinia sclerotiorum* consist of enzymes and various solutes, which have distinct temporal dynamics attributed to culture age and development. Hyphal exudates of *Fusarium culmorum* show definite distribution patterns in relation to colony morphology and vary in their appearance from transparent to granular and opaque.

However, clear scientific evidence on the function of droplets and their ecological role is lacking. It was argued that guttation droplets might function as a reservoir of water for maintaining constant growth of aerial hyphae. The droplets could mitigate suboptimal or stressful conditions due to unfavourable water potentials of cultivation media (Jennings 1991), but it also was suggested that guttations function as a reservoir of metabolic by-products, metabolite reserves, secondary metabolites or enzymes. Because exudates are possibly of cytoplasmic origin they also were linked to cell death and the rupture of cytoplasmic membranes in the development of sclerotia in *Sclerotinia* sp. and *Sclerotium* sp. (Georgiou et al. 2006). Exuded droplets might be the result of a selective excretion of cytoplasmic contents to cope with waste products. It was shown that high amounts of nutrients were readily reabsorbed after exudation while by-products such as oxalic acid were not. *Metarhizium anisopliae* is known to produce a number of toxic extrolites, such as destruxins, swainsonine, serinocyclins and cytochalasins (Krasnoff et al. 2007). Among those destruxins (dtx), chemically cyclic hepsadepsipeptides are the most abundant metabolites and predicted to have implications in fungal virulence (Hutwimmer et al. 2010). Apart from known insecticidal and phytotoxic activities, these compounds also have cytotoxic and antiproliferative effects on eukaryotic cells as well as suppressive effects on the hepatitis B viral surface antigen. Wang et al. (2004) suggested that the dtx synthesis genes are located on an extra chromosome, termed conditionally dispensable (CD). CD chromosomes have been found in plant pathogenic fungi and often contain genes coding for virulence determinants (Hutwimmer et al. 2010). Such results might be used to guide the in vitro production of destruxins for use in further research programmes or even as a possible biorational pest control agent. Mycorrhizae, a symbiotic association of certain fungi with the plant-root system, also increase the surface area for absorption of nutrients and water which is considered to increase the root pressure and there seems to be a relation between guttation and root pressure (Zholkevich 1992; Dustmamatov et al. 2004). On the other hand, mycorrhizal colonisation also leads to endophyte infection and endophytic fungi are exuded in the guttation fluid of infected grasses

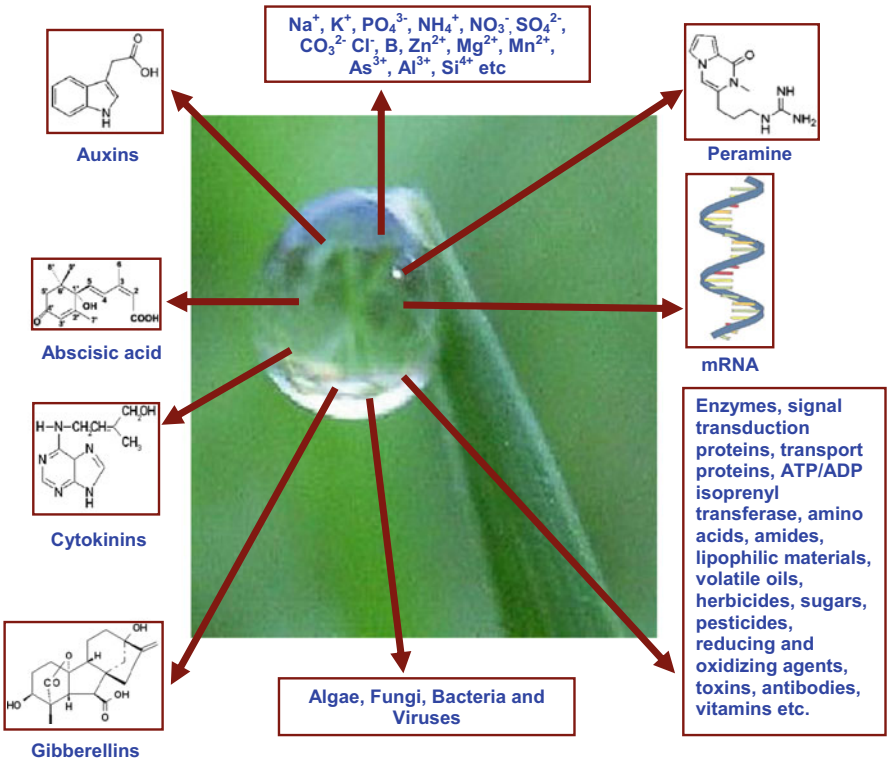

Fig. 4 Guttation fluid containing algae, fungi, bacteria and viruses in addition to a number of organic and inorganic compounds including metabolites, enzymes, hormones, vitamins, salts, ions, nutrients, etc. impacting plant behaviour (*Source*: Singh and Singh 2013)

(Liu et al. 2011). But further literature on the incidence of fungal diseases with respect to guttation in either field crops or horticultural crops including vegetables or cash crops like coffee, tea, etc. is conspicuously lacking. This might be due either to the lack of interest or this being no cause of concern.

4.5.3 Bacteriology of Guttation

When guttation occurs, the plant surface is wetted. These are the conditions that will allow epiphytic living motile bacteria to move and to eventually enter the plant's interior via the hydathodes. Guttation fluids have been found to carry a number of bacterial species with them. Smith (1898), who pioneered in the study of bacterial diseases of plants over more than a century ago, was the first to suggest a relationship between guttation drops on the periphery of a leaf and the possibility of infection taking place through these avenues of entrance into the host plant, and its subsequent spread through the tissues of the plant. In studying the black rot of cabbage, caused by

the bacterium *Xanthomonas campestris*, the author discovered and worked out thoroughly that guttation was the common approach of infection for this disease. Filtered and sterilised guttation fluids collected from grasses contained growth of a cnumber of soil microorganisms, including *Bacillus cereus*, *B. fluorescent, B. radicicola* and a nitrate reducer, which found great increase in their numbers after 24–48 h of incubation (Ivanoff 1963). Munnecke and Chandler (1957) found several types of bacteria and sooty moulds growing in the leaf exudates of the ornamental plant *Philodendron hastatum*. The abaxial surfaces of leaves developed 'exuding spots' which frequently showed necrotic centres. It was thought that the saprophytic bacteria associated with the spots may be responsible for the necrosis, although their role in the disorder was not demonstrated. The growth of *Xanthomonas oryzae* pv. *oryzae* (Xoo), causal agent of bacterial leaf blight and typical vascular pathogen, was investigated in tissues and guttation fluids of rice leaves to elucidate the mechanisms of susceptibility in the host–parasite interaction (Noda and Kaku 1999). Light and scanning electron-microscopic observations showed that, in the compatible cultivar–bacterial strain combination, bacterial masses were abundant in the lumen of xylem vessels, but were absent in other vascular elements. The inner surface of spiral and ring vessels where the bacterium multiplied appeared to be digested by the causal organism. Histochemical tests also revealed that the xylem vessel walls were degraded after infection with Xoo. Based on the histological study, unexpectedly, the growth rate of Xoo was very low in an aqueous solution of freeze-dried guttation fluid. When 0.5 % sucrose was added, however, the bacterium could grow extensively in a solution containing freeze-dried guttation fluids. The results suggested that nutritional factors such as pectic substances from affected xylem vessel walls were involved in the multiplication of Xoo in the lumen of xylem vessels. It seemed that the high sugar content of the exudate, ranging from 2.8 to 9.3 % aided by hormones, vitamins and other nitrogenous substances present therein, accelerated the abundant growth of the pathogen. However, much gap in our knowledge exists in the mode of bacterial infection and its spread associated with guttation water.

While studying the epidemiology of bacterial blight disease of rice, Rao and Srivastava (1970) found that the bacteria were also released in the guttation fluid of seedlings raised from infected seeds. *X. oryzae* could enter the vascular system of healthy rice seedlings through the roots and was detected in the guttation fluid. More recently, Carlton et al. (1998) studied and determined that hydathodes can serve as sites of entry and infection for the bacterial canker pathogen and can cause the development of marginal necrosis in tomato. The guttation fluids have been found to accumulate cationic peroxidase obtained from xylem vessels induced during incompatible interactions with *X. oryzae* pv. *oryzae* in rice (Young et al. 1995). The physiological mechanisms associated with resistance of cabbage to black rot disease seem to be associated with the hydathodes (Gay and Tuzun 2000). On the other hand, lignin deposition in and around the hydathodes was associated with the accumulation of a particular isozyme in hydathodal fluids. It is of practical significance to enquire whether the plants develop a protection mechanism against motile bacteria in the vicinity of the hydathodes. Such a protection mechanism could use the well-known pathogenesis-related (PR) proteins (Grunwald et al. 2003). The protein profile of the

guttation fluid was remarkably modified by treating plants with methyl jasmonic acid, suggesting that the protein composition of the guttation fluid is controlled by both internal and external stimuli. In an interesting development the age-dependent exudates of leaves have been shown to exhibit variable capacity for bacterial pathogenicity (Brandl and Amundson 2008). Thus, the leaf exudates appear to serve as powerful carrier of bacterial infections which is highly dependent upon the age of leaves. The sources of bacteria in these cases, however, have neither been confirmed nor their role when guttation fluid trickled down on the soil surface as well as the plant debris mulching the ground.

Differences in entry sites have been linked to distinct adaptive features and strategies in different plant pathogenic bacteria. Bacteria that commonly enter through natural entry sites where active basal defences are present, such as stomata and hydathodes (Melotto et al. 2006; Jackson 2009), may benefit from mechanisms such as coronatine-mediated stomatal opening which counteract these primary defences. The existence of both hydathode and stomata-specific entry strategies is supported by the observation that some pathogens preferentially enter plant leaves through different routes. For example, *X. campestris* pv. *campestris* preferentially enters leaves via hydathodes, while *X. campestris* pv. *Armoraciae* enters through stomata (Hugouvieux et al. 1998). Hydathodes, like stomata, are sites where nutrients and water may be released from the intercellular spaces, most notably in the form of guttation fluid, which may be exuded under conditions of high humidity and reabsorbed as the atmosphere and soil become drier, thereby drawing in hydathode-colonising bacteria. The composition of guttation fluid is distinctly different from apoplastic fluid, indicating that some nutrients are retained within the plant leaf, while others are exuded (Pilot et al. 2004). The hydathode entry mechanism of some bacteria, such as *X. campestris* pv. *campestris*, seems to require extracellular factors, such as extracellular polysaccharides and lipopolysaccharides, but is independent of the type-III secretion system, also called injectisome or injectosome, which is a protein appendage found in several Gram-negative bacteria (Gophna et al. 2003; Galan and Wolf-Watz 2006). One feature that may promote invasion through specific entry sites is attachment. Both *X. campestris* pv. *Hyacinthi* and *P. syringae* pv. *Phaseolicola* have been shown to preferentially attach to stomata (Romantschuk 1992). Conversely, bacteria that enter through wounds or active growth sites such as lateral root structures need chemotactic and nutrient utilisation abilities that allow them to move towards plant exudates and to competitively colonise infection sites (Brencic and Winans 2005; Yao and Allen 2006; Zolobowska and Van Gijsegem 2006). *Xanthomonas* is a large genus of Gram-negative bacteria that cause disease in hundreds of plant hosts, including many economically important crops. Pathogenic species and pathovars within species show a high degree of host plant specificity and many exhibit tissue specificity, invading either the vascular system or the mesophyll tissue of the host following their entry through plant's natural openings. Very recently, while studying the significance of guttation in the secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* in tomato greenhouses, Sharabani

et al. (2012) found that the exudation through guttation contributed to the formation of epiphytic populations on leaflets. This new knowledge may provide a simple and environmentally friendly means for decreasing the spread of the disease by avoiding contact with plants during periods when they bear guttation droplets. Of great interest the insights of host–pathogen interactions have been recently discussed at functional and comparative genomic levels which might encourage further research into bacteriology of guttation fluid (Ryan et al. 2011).

4.5.4 Virology of Guttation

Plant viruses are a class of plant pathogens that specialise in movement from cell to cell. As part of their arsenal for infection of plants, every virus encodes a movement protein, a protein dedicated to enlarging the pore size of plasmodesmata and actively transporting the viral nucleic acid into the adjacent cell (Schoelz et al. 2011). Like fungi and bacteria, particles of plant viruses in tomato (*Lycopersicon esculentum* Mill.), pepper (*Capsicum annuum* L.) and cucumber (*Cucumis sativus* L.) have been visualised by electron microscopy in guttation fluid of systemically infected plants (French et al. 1993; French and Elder 1999). As determined by enzyme-linked immunosorbent assay (ELISA), the concentration of *Tomato mosaic virus* (ToMV) in tomato guttation fluid was 0.9 ± 0.2 μg mL^{-1} and the concentration of *Pepper mild mottle virus* (PMMV) in green pepper guttation fluid was 0.5 ± 0.1 μg mL^{-1}. Of particular interest was the fact that viruses representing ten genera were visualised in infected cucumber (*Cucumis sativus* L.) plants. Recently, under guttation-promoting conditions, surface guttation fluid from *Brome mosaic virus* (BMV)-infected barley, wheat and maize plants was analysed for the presence of the virus by biological and serological assays (Ding et al. 2001). The authors detected virus particles in guttation fluid accompanied by initial viral symptoms in leaves showing either systemic necrosis or chlorotic streaks in all of these cereal crops. They proposed that in infected barley leaves, BMV exits from damaged vein cells (especially the xylem elements), accumulates in intercellular spaces and then reaches the surface of the leaves through water stomata during guttation or transpiration. Further, the roles of guttation fluid, irrigation water, contact between plants and transplantation into contaminated soil in the transmission of *Rice yellow mottle virus* (RYMV) have been assessed and detected in infected plants by ELISA and by inoculation to susceptible rice cultivar BG90-2 (Traore et al. 2008). Transmission tests from this fluid led to high disease incidence (87 %). The authors concluded that although guttation fluid is highly infectious its contribution to virus infectivity in irrigation water is negligible as field-irrigation water was not found to be an infectious source for RYMV. However, further experiments limiting the virus inoculum source to guttation fluid are necessary to prove that this fluid is an inoculum source.

4.6 Plant Defence Against Pathogen Attack

4.6.1 Preformed Chemical Defences

Chemicals such as phenolics, tannins, short-chain fatty acids, glycosylated steroids, unsaturated lactones, sulphur-containing compounds and terpenoids are known to be effective inhibitors of fungal and bacterial growth. It, therefore, holds promise for crafting agricultural and horticultural including cash crops like coffee, tea, cocoa, etc. through genetic engineering for enhanced production of such effective inhibitors of fungi and bacteria enhancing crop productivity.

4.6.2 Infection-Induced Physical Factors

Some physical factors that can influence pathogen establishment in plant tissues include the development of lignified cell walls, the formation of papillae that resist the penetration of fungal pegs, the formation of cellulose sheaths impregnated with toxic phenolics that surround fungal hyphae, the formation of tyloses in xylem elements which restricts the movement of pathogens and the production of gums and resins that seal off the pathogen from nutrients needed for growth. It is, therefore, obvious that host plants have ability to create and use a variety of barriers against entry and development of pathogens.

4.6.3 Infection-Induced Chemical Factors

Resistant species of plants may respond to pathogens by engaging the hypersensitive reaction, which is a series of rapid chemical responses induced in the plant by the pathogen that effectively isolates the pathogen from healthy tissues and produces substances that will inhibit or kill the invading organism. Phytoalexins are a diverse group of chemicals consisting of terpenoids, flavonoids and isoflavonoids that can subsequently be induced in the host by the pathogen and can inhibit the growth or kill the pathogen. Other chemical responses of plants to pathogen attack include the formation of pathogenesis-related proteins (i.e. chitinases, lysozymes, osmotin-like proteins, glucanases, proteinases, proteinase inhibitors, chitosanases and peroxidases). Both host and pathogens can detoxify various chemicals by directly metabolising such molecules or by forming nontoxic conjugates resulting in protection of host plants from infection.

4.7 Guttation and Disease Resistance of Crop Plants

Guttation fluid also serves as vehicle for the inhibition of disease-causing fungal, bacterial and viral growth due to toxins, mycotoxins, alkaloids, etc. produced by these

microorganisms. As stated earlier, Lewis (1962) studied the susceptibility of three cereals to *Claviceps purpurea* using their *g*uttation fluids as media for the growth of germinating spores of this fungus. Rye guttation fluid produced the most growth and barley fluid the least. The degree of susceptibility was correlated to the variation in the production of organic and inorganic compounds including vitamins by these crop species (Goatley and Lewis 1966). Recently, signals involved in host–microbe interactions have been detected (Brencic and Winans 2005; Ryan et al. 2011). High amounts of mycotoxins could be excreted from toxigenic *Penicillium* isolates into guttation droplets which could account for the variation in the intensity of disease incidence (Gareis and Gareis 2007). Secretion of antimicrobial phylloplane proteins by specific trichomes and delivering in guttation fluid from hydathodes protecting plants from diseases have been reported (Shepherd and Wagner 2007). Also, guttation has been demonstrated to reduce wood moisture, thereby preventing wood initial decay by fungus (Schmidt and Czeschlik 2006). Guttation fluid has also been used to differentiate varietal resistance to bacterial diseases (Fukui et al. 1996). Growth and survival of *X. campestris* pv. *Dieffenbachiae* in guttation fluids, i.e. xylem sap exuded from leaf margins of *Anthuriums*, have been found to be suppressed by several bacterial strains indigenous to leaves of various *Anthurium* cultivars. Growth and survival of *X. campestris* pv. *Dieffenbachiae* were monitored in guttation fluids of various *Anthurium* cultivars with a bioengineered, bioluminescent strain (V108LRUH1). The population of V108LRUH1 progressively decreased from 6.4 log CFU/mL (initial density) to <1.0 (not recovered) in some fluids but only to 5.0 log CFU/mL in others during 14 days. The decline was not correlated with cultivar susceptibility. Guttation fluids normally contained 5–8 dominant bacterial species with population densities ranging from 6.8 to 7.5 log CFU/mL. Five bacterial species were isolated from two highly inhibitory guttation samples and examined for inhibitory effects against V108LRUH1 in filter-sterilised guttation fluid. A mixture of the five species (6.5–7.0 log CFU/mL for each) was highly inhibitory to V108LRUH1, although the individual species showed little or no inhibitory activity. Removal of one species either nullified or had no effect on inhibitory activity depending on the species removed from the mixture, suggesting that the specific combination of bacteria in guttation fluid determined its inhibitory effect. When inoculated onto *Anthurium* plants 1 day prior to inoculation with the pathogen, the mixture of five species was more effective in reducing severity of leaf infection than individual strains.

Fukui et al. (1999) extended their work further to investigate the effect of single versus multiple biological control agents (BCAs) on suppression of bacterial blight of *Anthurium* using a bioluminescent strain (V108LRUH1) of *X. campestris* pv. *Dieffenbachiae*. When five BCAs (GUT3, GUT4, GUT5, GUT6 and GUT9) were co-inoculated in various combinations with V108LRUH1 into filter-sterilised guttation fluids of *Anthurium* plants, a mixture of all five strains or four strains without GUT9 was most inhibitory to V108LRUH1. None of the individual BCAs inhibited V108LRUH1 in the guttation fluid. When BCAs were sprayed at congruent with 10(8) CFU/mL on the foliage of a susceptible cultivar 1 day prior to inoculation with V108LRUH1, GUT6 alone and any mixtures containing GUT6 were highly effective in suppressing wound invasion and subsequent leaf infection

by V108LRUH1. When tested on several cultivars that differed in susceptibility to the disease, the mixture of five strains or four strains without GUT9 consistently suppressed leaf infection regardless of the cultivars. In some cultivars, BCAs completely suppressed both wound and hydathode invasion by V108LRUH1, resulting in no infection in many leaves. These results indicate that application of bacterial mixtures provides *Anthurium* cultivars with bacterial communities suppressive to *X. campestris* pv. *Dieffenbachiae*. The results also suggest that selecting an effective mixture of BCAs first and then removing ineffective strains may be a better general approach to finding the most effective BCAs than finding individual strains and combining them. Inhibition of growth was not observed in filter-sterilised guttation fluids and was restored to original levels only by reintroducing specific mixtures of bacteria into filter-sterilised guttation fluids. These findings are of great significance as this bacterial community has the potential for biological control of *Anthurium* blight.

The epithem can also add substances to the guttation fluid, such as excess calcium and proteins that protect vulnerable hydathodes from microbial and fungal attack. As stated earlier, when guttation occurs, the plant surface is wetted. These are the conditions that will allow epiphytic living motile bacteria to move and to eventually enter the plant's interior via the hydathodes. Hydathodes of *Arabidopsis thaliana*, which are capable of protein synthesis, have been implicated in the secretion and retrieval of ions and organic solutes from leaf surfaces (Pilot et al. 2004). Therefore, hydathodes function in both the extrusion and collection of water and nutrients. Pathogenic bacteria such as *X. oryzae* exploit this phenomenon to attain entry into leaf interiors. The defensive enzyme cationic peroxidase has been found in guttation fluid of rice during infection with the *X. oryzae* (Young et al. 1995). Guttation fluid collected from healthy leaves of barley contains pathogenesis-related proteins so plants have mechanisms to guard against such intrusions (Southworth 2012). Grunwald et al. (2003) brilliantly investigated as to whether the plant has capacity to develop a protection mechanism against motile bacteria in the vicinity of the hydathodes. Such a protection mechanism could use the well-known pathogenesis-related (PR) proteins. Indeed, an analysis of the guttation fluid showed a clustering of approximately 200 proteins which belonged mostly to the family of PR-proteins suggesting a role in plant protection against invaders. The protein profile of the guttation fluid was remarkably modified by treating plants with methyl jasmonic acid, suggesting that the protein composition of the guttation fluid is controlled by both internal and external stimuli. The anti-POC1, an antibody, reacted only with a protein of the same mobility as POC1 in extracellular and guttation fluids from plants undergoing incompatible responses. In an effort to understand the physiological mechanisms associated with resistance of rice to *X. oryzae* pv. o*ryzae* (Young et al. 1995) and resistance of cabbage to black rot disease (Gay and Tuzun 2000), the roles of hydathodes in disease resistance with respect to total peroxidase activities, anionic peroxidase isozyme expression and lignin deposition were investigated. Hydathodal fluids of resistant varieties had greater peroxidase activity when compared to susceptible ones, with infected plants having higher peroxidase levels than noninfected plants. Lignin deposition in and around the hydathodes was found to be associated

with the accumulation of this particular isozyme in hydathodal fluids. Thus, it is of immense significance that guttation fluid can be used to differentiate varietal resistance to fungal as well as bacterial diseases.

Based on the sequence data for different isoforms it would be possible to produce isoform-specific antibodies and to design specific primers. It has been shown that some isoforms of pathogenesis-related proteins (PRs) possess antifungal activity. It would be interesting to study where different isoforms are localised and how they are coordinated in the plant defence system. A tissue-, time- or pathogen-specific expression and localisation study on different isoforms could shed more light on their specific functions. There are cultivar differences in barley resistance towards *Bipolaris sorokiniana*. These genetic differences play an important role in the early crucial recognition of a pathogen and the subsequent timing of defence responses in a plant. In these studies changes in mRNA accumulation occurred within the first 6 h. It would be interesting to compare susceptible and resistant cultivars in the early stages of infection by guttation to see where and when transcribed mRNA and the subsequent PRs are located in correlation with adhesion, penetration attempt and hypersensitivity response. From the foregoing discussion it seems increasingly clear that guttation fluid plays important role in defence against various bacterial and fungal diseases and that it also elevates disease resistance in plants.

4.8 Fungal Secretions of Guttation as Defence Against Herbivores and Insects

Plants produce a wide variety of substances which at first sight seem to be excretory products. However, many of them have been found to have a function, for example, in defence against herbivores and parasites. The accumulation of toxic compounds at the surface allows their direct contact with insects, pathogens and herbivores. Many grasses live in association with asymptomatic fungi (*Neotyphodium* spp. endophytes), which grow in the intercellular spaces of the grass. These endophytes produce a range of alkaloids that protect the grass against grazing by mammals and insects. Endophytic fungi, such as *Neotyphodium* sp. (Ascomycota), which asymptomatically colonise in intercellular spaces of grasses, produce a range of alkaloids that can be extruded in guttation fluid and act as deterrents to herbivory (Koulman et al. 2007, 2012). Hydathodes secrete inorganic ions such as Na, Cl, Ca, Cd, Zn, Mn, Ni, Pb, S and Si under certain conditions; salts of these elements sometimes form crystals on hydathodes and trichomes (Fig. 2) (Wagner et al. 2004; Chen and Chen 2005). A gene encoding a boron efflux transporter was shown to be a significant component of boron tolerance in barley, as high expression of this gene at leaf tips containing hydathodes correlated positively with the presence of boron in guttation fluid (Sutton et al. 2007). These observations suggest that rather than simple pores, hydathodes are complex and variable phylloplane structures with diverse roles.

A good report showing the mobilisation of fungal alkaloids into plant fluids by the host plant in grass–endophyte associations has been prepared (Koulman et al. 2007). One of these alkaloids is an unusual pyrrolopyrazine, peramine. Peramine appears to be continuously produced by the endophyte, but does not progressively accumulate. No mechanism for the removal of peramine by its further metabolism or any other process has been reported. Peramine was detected in the cut leaf fluid as well as in the guttation fluid of all grass–endophyte associations. In some associations the lolines and ergot peptide alkaloids have also been detected. Thus it seems that the guttation and trichome exudates are ideally positioned to provide a first line of defence against attacking organisms, perhaps providing time for activation of induced defences (Wagner et al. 2004). Shepherd and Wagner (2007) have described the physical structures and biochemicals of the phylloplane and discussed protein-based surface defences of animals. They have also reviewed the emerging evidence pertaining to antimicrobial phylloplane proteins and mechanisms by which proteins can be released to the phylloplane, including biosynthesis (e.g. phylloplanins) by specific trichomes and delivery in guttation fluid from hydathodes. Gareis and Gareis (2007) found eight of eleven ochratoxigenic isolates of *Penicillium nordicum* and *Penicillium verrucosum* produced in guttation droplets when grown on Czapek yeast extract agar (CYA) for 10–14 days at 25 °C. This shows that high amounts of mycotoxins could be excreted from toxigenic *Penicillium* isolates into guttation droplets which could account for the variation in the intensity of disease.

4.9 Chemicals of Guttation as an Aid to Fungal Classification

It is important to point out that guttation fluid may provide an excellent opportunity for studying host–parasite relationship in plants. The colour of guttation droplets is often crystal clear and translucent. However, it can vary depending upon the strains, races and forms of fungi invading leaf tissues. The colour and intensity of guttation fluid have been used for taxonomic classification and utilised to identify and distinguish various strains, races and forms of Aspergilli and Pencillia (Frisvad et al. 2004). By using this technique, Raper and Thom (1949, 1968) examined hundreds of isolates of the *P. chysogenum* 'series' and considered *P. griseoroseum* Dierckx to be a synonym of *P. notatum*, and Samson and Gams (1984) reinforced the broad concept of *P. chysogenum* by further reducing *P. cyaneofulvum, P. meleagrinum* and *P. notatum* to synonymy with *P. chysogenum*. Recently, Scott et al. (2004) observed yellow guttation droplets and they utilised colour variations as the basis of classification and categorisation of *Penicillium* sps which may aid greatly the control measures for the disease caused by this fungus. However, little is known about the composition of such exudates. Further studies may look for other disease-causing fungi or otherwise producing variants of colours to be used for crop protection purposes.

Summarily, from the foregoing discussion of the microbiological aspects of guttation it amply reflects that guttation seems to provide conditions conducive to incidence of certain fungal, bacterial and viral diseases in plants. However, much remains to be done on genotypic variability in chemical constituents of guttation including toxins, hormones and enzymes and their interactions involving wild types, mutants and transgenic lines of crop plants. Paucity of research work also exists at genomic and molecular level for the initiation and development of hydathodes, their distribution, hydathodal index and its relation to disease incidence, the involvement of structural integrity of hydathode in the production and retention of hormones, nutrients and toxins and their contribution to disease incidence or resistance, etc. It is hoped that in future these gaps in knowledge and those described in earlier paragraphs will serve as stimulant for further research.

5 Outlook

The microbiology of guttation including its phycology, mycology, bacteriology and virology has been highlighted in this review. The light radiating from new findings makes it amply clear that guttation does provide conditions conducive to incidence of certain fungal, bacterial and viral diseases in plants of economic importance. A number of newly synthesised pathogenesis-related proteins (PR-proteins) following host–parasite interactions upon entry into the plant tissues of pathogens present in the guttation fluid have been recently identified, partially characterised and implicated in possible strategies evolved by plants to fight against pathogenic infections. However, much remains to be done on genotypic variability in chemical constituents of guttation fluid including toxins, hormones, vitamins and enzymes and their interactions involving wild types, mutants and transgenic lines of crop plants. A high degree of vacuum also exists in research at genomic and molecular level with respect to initiation, development and distribution of hydathodes in relation to disease incidence and resistance. Studies on the structural integrity of hydathodes in the production and retention of hormones and their interactions with toxins in disease incidence or resistance are also lacking. Further, there is absolutely no information on the QTL mapping for guttation efficiency of various genotypes of different guttating species of crop plants which, hopefully, should be possible now with the invention of the new technique for measuring guttation developed in authors' laboratory in India to construct new plant types for increased crop productivity in order to achieve food and nutrition security across the world. I hope that in future these gaps in knowledge and those described in earlier paragraphs of this review will tease the readers for future research leading to the creation of genetically transformed plants with increased production and secretion of peptides to kill the pathogens facilitating eco-friendly and sustainable agriculture system worldwide.

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Adaptation of Rice to Flooded Soils

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Abstract This paper and its companion (Colmer et al., 2014) review research on the adaptation of rice (*Oryza sativa* L.) to the wide range of semi-aquatic environments in which it grows. The paper considers well-regulated flooding to 5–20 cm depth; the companion considers deeper flooding in rainfed conditions. Flooded environments are dominated by the very slow diffusion of gases in water and the resulting changes in soil chemical and biological conditions. Adaptations to these potentially toxic conditions hinge on an optimum ventilation network in the plant, providing O_2 to the roots and rhizosphere, both being critical for favourable nutrition and tolerance of reduced-soil toxins. Rice has become a model for studying adaptation to flooded soils and flood-prone environments because of its relatively simple genome and large genetic diversity, and its extreme tolerance of flooded soils compared with other crop species.

1 Introduction

The review focuses on the mechanisms of adaptation of rice (*Oryza sativa* L.) to wetland environments, where the slow diffusion of gases in water results in greatly altered plant growth conditions. These mechanisms have been studied for many decades by plant physiologists, biochemists, and biophysicists, as well as by soil chemists, biologists, and physicists. Concurrently, there have been intensive agronomic and genetic studies, and rice breeding. These streams are increasingly coming together, particularly with the application of molecular genetics and bioinformatics. The great genetic diversity and relatively simple genome of rice have made it well suited to research into the mechanisms of plant adaptation to flooded environments.

This endeavour has been stimulated by the role of rice as a staple food for millions across the globe. It is the only major food crop that is cultivated in a semi-aquatic environment. Under controlled flooding and optimal management, the potential grain yield of rice is equal to that of wheat (10–11 tons ha^{-1} in its main agro-ecological zones—Fisher and Edmeades 2010). Rice has become one of the best explored model systems for adaptation to flooded environments. Its genome contains a multitude of adaptive mechanisms which have evolved naturally and through intense human intervention.

1.1 Rice in Wetland Ecosystems

Flooded environments are dominated by the influence of Fick's first law of diffusion: $F_i = -D_i \, dC_i/dx$, where F_i is the flux of gas or solute i in a medium in which it has concentration gradient dC_i/dx and diffusion coefficient D_i. Diffusion coefficients in water are 10,000 times smaller than those in air (Armstrong 1979). So rates of diffusion of respiratory gases into and out of flooded soils are extremely slow. Thus, the flooded environment is a 'different world' to that of the well-aerated soils in which all other food crops are grown.

The diverse ecology of wetlands used for agriculture has been reviewed by Verhoeven and Setter (2010). Here we focus on rice and as appropriate we include other species with particular tolerance mechanisms. Rice is grown in water depths ranging from 5 to 20 cm in irrigated or rainfed paddy fields, to 0.5 to over 5 m in flood-prone areas, with floods lasting weeks to months. All these environments impose very restricted gas exchange on the submerged parts of the plant and low to zero O_2 concentrations in the surrounding soil.

Few plant species offer the opportunity to understand adaptation to low O_2 environments. Rice has been a model for investigation because of its significance as a food source and the resulting extensive collection and documentation of germplasm that have taken place. Though most modern rices have a common ancestry, many thousands of landraces have been catalogued from a wide range of flooding regimes.

Rice shares its ability to thrive in inundated regions with a large number of other wetland plant species (Blom and Voesenek 1996). Other species of interest include *Echinochloa* (barnyard grass), whose subspecies include some of the few effective weeds of rice, possibly with superior ability to grow in anoxic soils (Kennedy et al. 1980; Ismail et al. 2012). Other wetland species that have been studied include *Phragmites australis*, which ventilates its rhizomes via pressure flow as well as by diffusion (Armstrong et al. 1992); some wild *Oryza* species are perennials, but it is not clear whether these have a similar ventilation system to *Phragmites*. Another intensively studied species is *Rumex palustris*, a dicotyledon native to European flood plains; different *Rumex* species flourish in locations of very different water regimes over short distances (Blom and Voesenek 1996). Others include various water weeds, the best researched of which are *Potamogeton* species which have turions that can survive for long periods in anoxic mud and develop shoots even under anoxia (Jackson and Ram 2003; Ishizawa et al. 1999). Responses of these species relevant to the present review are discussed in the appropriate sections. A more general review is given by Colmer and Voesenek (2009).

1.2 Early Research on Rice Physiology and Soil Chemistry

This short historical account covers both the present and its companion paper. Pioneering studies were on the ventilation essential to efficient root function, but also to oxygenation of the rhizosphere to facilitate nutrient uptake and to protect the

roots from the toxic compounds in the anoxic soil. This included ingenious studies initiated during the Second World War by MH van Raalte in the Botanical Gardens of Bogor in Java (van Raalte 1940, 1944). His results stimulated subsequent intensive investigations by Armstrong and colleagues, in which experimental observations were combined with modelling of O_2 diffusion from the shoots to the roots and to the rhizosphere (Armstrong 1979; Armstrong and Beckett 1987).

Tolerance to anaerobiosis was a second focus, with concentration on the ability of germinating rice seedlings to develop a coleoptile (acting as a snorkel), even during anoxia. Initially these studies were on mechanisms of extension growth (Kordan 1974) and then complemented by biochemical investigations (Bertani and Reggiani 1991; Perata and Alpi 1993; Ricard et al. 1994; Gibbs and Greenway 2003; Greenway and Gibbs 2003). Germinating rice became a productive model system to elucidate mechanism of anoxia tolerance in plants. Functions of the phytohormone ethylene were another research avenue (Kende et al. 1998; Colmer et al. 2013).

The other main strand of research was on soil conditions, with emphasis on redox reactions and their effects on nutrient availability and the concentrations of toxic compounds (reviewed by Ponnaperuma 1972; Kyuma 2003; Kirk 2004).

The advent of molecular biology and the sequencing of the genomes of both *indica and japonica* rice subspecies (Doi et al. 2008) has rejuvenated these earlier endeavours. A logical dovetailing of genomic research with physiology, agronomy, and plant breeding has occurred, integrating with molecular genetics techniques.

1.3 Outline of the Review

The two parts of this review concern the mechanisms of adaptation of rice to its large range of environments, with special reference to the water regimes. We discuss the intriguing features of rice adaptations to this hostile environment with emphasis on aeration, gas exchange, nutrient uptake and tolerance to toxins, and on the relevant biophysics, biochemistry and molecular biology. In the present paper, we describe the ventilation network and the chemistry and physics of flooded soils. The second paper (Colmer et al. 2014) concerns deeper floods and considers water quality, underwater gas exchange, and elongation in deep water and longevity during transient complete submergence. A third paper in preparation by some of the present authors will consider the metabolic mechanisms of anoxia tolerance, for which germinating rice, particularly the coleoptile, has become an excellent model.

2 Characteristics of Rice Ecosystems

2.1 Geography

The general features of rice flooding regimes depend on the regional and local climate, and the soils and landforms, but modified to a great extent by artificial levelling and bunding of individual fields, and irrigation (Moormann and van Breemen 1978; Richardson and Vepraskas 2001). The main landforms are inland valleys in upland areas, alluvial fans and terraces in foothills, active floodplains in river basins, and coastal floodplains. There are three distinct types of hydrology in these landforms, depending on the position in the landscape: *fluxial* lands, in which water arrives wholly or in part from surface flow, such as in runoff or streams; *phreatic*, in which water arrives from groundwater that rises to the soil surface for at least part of the year; and *pluvial*, in which water arrives entirely from rainfall. In fluxial lands, water flowing in from neighbouring upland and upper catchments brings with it sediment and nutrients which are only slowly lost to deepwater areas downslope. Because of the net inflow of nutrients, the abundance of water, and beneficial changes in the soil resulting from chemical reduction under anoxia (Sect. 3.2), fluxial wetlands are among the most productive ecosystems on Earth. By contrast pluvial wetlands rely on nutrients brought in by rainfall or fixed biologically from the atmosphere, and they therefore tend to be much less productive. Phreatic wetlands are intermediate.

2.2 Water Regimes

The terminology most widely used to define rice ecosystems is based on water regime (deficit, excess, or optimum), drainage (poor or good), topography (flat or undulating), and soil characteristics (with or without problems) (IRRI 1982). Four major categories are distinguished as illustrated in Fig. 1.

2.2.1 Irrigated

Roughly 55 % of the harvested rice area is irrigated, producing 75 % of the world's rice (IRRI 2002). Irrigated rice is grown in levelled, bunded fields with good water control. The crop is transplanted or direct seeded in puddled soil, and shallow floodwater (5–20 cm deep) is maintained on the soil surface so that the soil is predominantly anoxic during the rice growing season. One or more crops are grown each year. In irrigated wet-season rice, water may be added as a supplement to rainfall, generally early in the season or during mid-season dry periods. In dry-season rice,

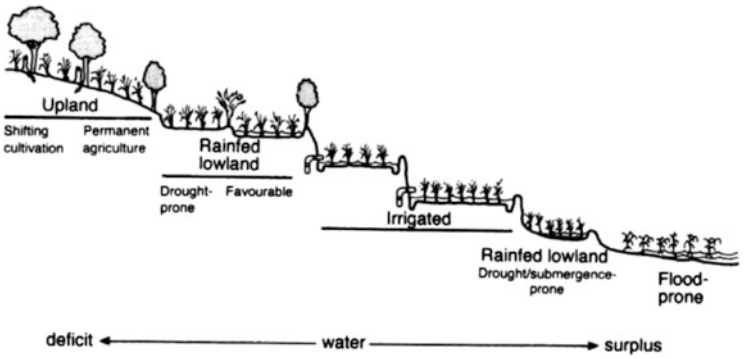

Fig. 1 Characteristics of rice ecosystems (IRRI 1993). Rice ecosystems are characterised by water and land resources. Irrigated rice can occur at any point in the toposequence; the others occur as shown. See text for definitions. Reproduced by permission of IRRI

crops cannot be grown without irrigation. Cloud cover is minimal and therefore solar radiation and hence yield potentials are greater than for wet-season rice.

2.2.2 Rainfed Lowland

Rainfed lowlands account for roughly 35 % of the harvested rice area globally but less than 20 % of production (IRRI 2002). The rice is grown in level to gently sloping, bunded fields that are flooded for at least part of the cropping season. Water depths may exceed 20 cm. The crop is transplanted in puddled soil or direct seeded on puddled or ploughed dry soil. The water supply is poorly controlled, with the soil alternating between aerated and anoxic conditions, and both transient submergence of the plants and drought may occur in the same season, requiring different varieties and management strategies to irrigated systems.

2.2.3 Flood-Prone

Flood-prone areas account for less than 5 % of the global harvested rice area (IRRI 2002), but where they occur they may be vital for the local economy. They are distinguished from rainfed lowlands by the depth (>50 cm) and duration (between a few days and 3 weeks) of complete submergence in flash-flood areas, to deep partial flooding in stagnant and deepwater areas, or by soil problems related to flooding (e.g. salinity, sodicity, acid sulphate, peat soil). Fields are flooded to at least 50 cm depth and often much more for periods from weeks to months. Varieties must be adapted to flash floods where the water rises rapidly but drains within 2 weeks, or to more gradual but prolonged flooding to depths exceeding 100 cm, requiring the

plant to elongate to reach the surface (deepwater/'floating' rice of over 5 m long is also represented here).

2.2.4 Upland

Upland rice accounts for only a few per cent of global production (IRRI 2002). It is grown in level to steeply sloping fields that are rarely flooded or waterlogged. No effort is made to impound water as for other rice ecosystems and the crop is direct seeded on ploughed dry soil or dibbled in wet, non-puddled soil.

2.3 Genetic Diversity of Rice

The genus *Oryza* originated about 130 million years ago from a common ancestor followed by introgressions of wild marsh grasses into modern rice (Khush 1997) domesticated around 9,000 years ago in Asia (Molina et al. 2011). Two distinct subspecies of *O. sativa*, *japonica* and *indica*, arose from independent populations more than 100,000 years ago (Sweeney and McCouch 2007), followed by a series of introgression, selection, and diversification events that occurred naturally before human intervention. Subsequent changes to the genetic composition of *O. sativa* have been enhanced by human activity (Vaughan et al. 2005) to produce some half a million identifiable landraces and commercial varieties of *O. sativa*, of which about 20 % are curated at the International Rice Research Institute (Sackville-Hamilton 2006). The contribution of continuing introgression to rice evolution has been argued (Ge and Sang 2007), but regardless of the accepted model, rice remains a dynamic species whose genetic improvement will accelerate through biotechnology (Hirochika et al. 2004; Jung et al. 2008). Within *O. sativa*, vast genetic potential remains to be exploited (Xu et al. 2012).

Relatively few rice genotypes have been investigated at the gene level, despite the *japonica* genome having been mapped more than a decade ago and in spite of intensive genomics addressing key evolutionary questions (Ge and Sang 2007). A seminal study of individual genes including a shattering gene and a pericarp colour gene has been used to establish domestication of *O. sativa* from various progenitors that include *O. rufipogon* and *O. nivara* (Sweeney and McCouch 2007). With this significant speciation, we speculate that many genes that confer tolerance to flooding regimes remain in modern rice cultivars. Even though at most 20 % of genetic diversity in the genus *Oryza* is found within the two subspecies of *O. sativa* (Zhu et al. 2007), enormous potential for tolerance to flooding regimes exists, as demonstrated in this review. This is particularly the case because of the diverse flooded habitats to which most rice species are habituated.

In addition to *O. sativa* and *O. glaberrima*, which have been domesticated for thousands of years in Asia and Africa, respectively, there are more than 20 wild *Oryza* species that remain largely unexploited. Six species share the AA genome

with the two domesticated rice species and are therefore candidates for genetic improvement of cultivated rice (Sweeney and McCouch 2007; Doi et al. 2008; McNally et al. 2009). Reciprocal crosses reveal a cytoplasmic contribution to fertility that will need to be accommodated if wild germplasm is to enrich domesticated rice, with *O. nivara* the most compatible species with *O. sativa* (Naredo et al. 1997). Species such as *O. rufipogon* which grow as perennial weeds in wetlands are potential sources of flood tolerance genes, especially for deepwater conditions. Crosses between *O. sativa* and *O. rufipogon* demonstrate the potential for the wild relative as a donor of yield-related genes (Septiningsih et al. 2003; Sabu et al. 2009); this heritability gives hope for transfer of flood tolerance genes from the *O. rufipogon* gene pool.

Identification and transfer of genes conferring flood tolerance from the wild *Oryza* species to domesticated rice will, however, require exhaustive genomics approaches and cytogenetic techniques to facilitate introgressions of small chromosome regions containing critical genes or target QTLs (Doi et al. 2008). The search for useful alleles will accelerate as collections of wild rice relatives are screened and reproductive barriers are surmounted (Doi et al. 2008). Rapid genetic progress will specifically come from a deeper understanding of transcriptional control, as demonstrated with the *Sub1A* gene (Xu et al. 2006). Mazzucotelli et al. (2008) point out the importance of transcriptional regulation in developing genotypes that can tolerate thermal stresses and drought. Liu et al. (2010) report a number of anaerobically responsive motifs in *O. sativa* that are candidates for transcriptional control of flood tolerance genes. As critical QTLs for flood tolerance and the identities of key alleles are identified in *O. sativa*, tolerance genes will also be revealed in wild relatives, particularly as genome sequences become public. Specifically, improved tolerance of domesticated *Oryza* species to contrasting flooding regimes will require consideration of an array of gene × environment interactions. There will also be benefits for flood tolerance in more susceptible cereals because of the synteny that allows targeting of genes in related grass genomes (Xu et al. 2012).

3 Characteristics of Flooded Environments

3.1 Impaired Gas Transport

In all rice ecosystems, except upland, there is standing water on the soil surface for most of the growing season (Fig. 2). In irrigated rice, the water depths range from 5 to 20 cm. In rainfed rice much greater depths occur during flooding, and sometimes these floods last several months, in which case deepwater rices are grown. The concentrations of dissolved gases in the floodwater depend on the growth and respiration of photosynthetic algae, aquatic weeds, and non-photosynthetic organisms in the water (Roger 1996). Roger (1996) found that

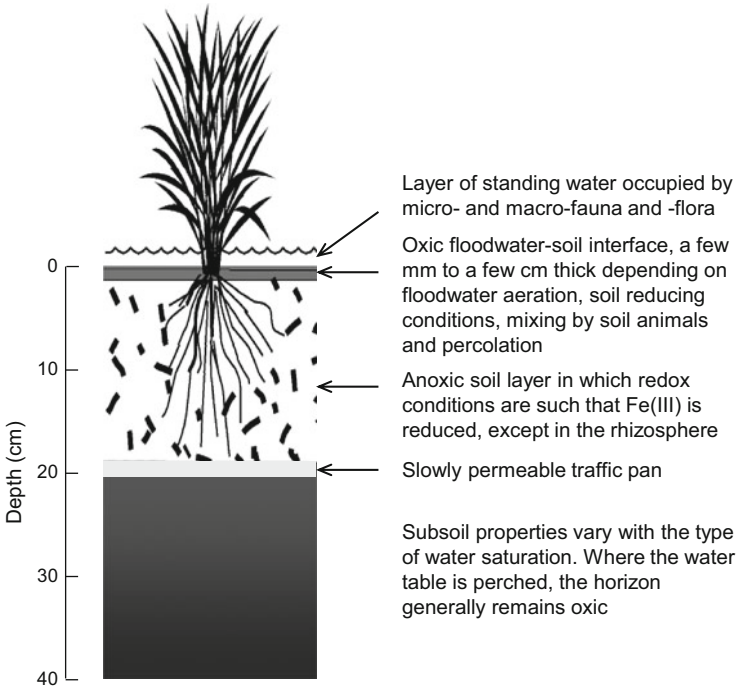

Fig. 2 Schematic profile of a flooded rice field (Kirk 2004)

in irrigated rice fields with 15 cm water depth photosynthesis can lead to O_2 concentrations of 0.31–0.62 mM (34–68 kPa), while at night, dissolved O_2 concentration can fall to near zero. Opposite diurnal cycles occur for CO_2, with associated changes in pH, which increases when CO_2 decreases and vice versa. For example, Mikkelsen et al. (1978) found the floodwater pH in an irrigated rice field rose as high as 10 during the day as CO_2 was removed but fell by 2 or 3 pH units at night.

In the soil beneath the floodwater, low rates of diffusion and the absence of turbulence cause O_2 concentrations to drop to zero within a few hours of flooding as O_2 is consumed by plant tissues and microorganisms (Ponnaperuma 1972). Anaerobic soil microbes then use alternative electron acceptors in their respiration. Dissolved CO_2 formed in the respiration of these organisms escapes only slowly, so it tends to accumulate. Ponnaperuma (1972) reported CO_2 pressures of 18–38 kPa in flooded soils without plants at 3 weeks after the start of flooding. There are also increases in ethylene: for example Trought and Drew (1980) found ethylene reached 0.4 Pa in a waterlogged wheat soil and Setter et al. (1988) found ethylene reached 2.5–4.5 Pa in soils in deepwater rice fields.

3.2 Changes in the Soil Following Flooding

3.2.1 Soil Physical Changes

Following flooding, air trapped in soil pores becomes compressed and further compression develops as volatile products of organic matter decomposition accumulate in the pores and as 2:1 type clays swell (Greenland 1997). As a result, large soil aggregates tend to rupture, and further rupture occurs as organic matter and oxide coatings that bind aggregates dissolve. The soil permeability declines as disaggregation proceeds and as pores become clogged with dispersed clay and other debris.

The practice of puddling the soil during land preparation for rice causes further disaggregation. The aim of puddling is to reduce losses of water through percolation, both to conserve water and to control weeds, and to facilitate transplanting. Puddling results in near complete destruction of water-stable aggregates and dispersion of fine clay particles. Some flow-through of water should be maintained so that the soil does not become entirely anoxic and toxins are washed out. Also, if the structure is completely destroyed the soil will dry only very slowly following the rice crop, and this will delay the establishment of a following dryland crop. Puddling decreases percolation rates by up to three orders of magnitude. It generally leads to an increase in total porosity because the destruction of aggregates decreases intra-aggregate pores but increases inter-aggregate and inter-domain pores. In the absence of puddling, the flooding of intra-aggregate pores results in large volumes of anaerobic conditions within aggregates, while near atmospheric O_2 concentrations occur between the aggregates when the soil drains.

Repeated working of the soil for rice often results in permanent physical changes that mask the soil's original character. Gross changes are caused by levelling, terracing, and puddling of the soil. Over time a 'traffic' pan of compacted soil often develops, 5–10 cm thick at 10–40 cm depth. This has a greater bulk density and is less permeable than the overlying surface soil, but has similar texture. Over time, the surface soil often becomes more coarse-textured, possibly because of weathering of clay under alternating flooding and drainage (Moormann and van Breemen 1978). Clay may also be lost from the surface during puddling by movement downslope with surface water. But equally clay may be added from upslope.

3.2.2 Soil Chemical and Biological Changes

Because O_2 is excluded by submergence, soil organisms must use other soil constituents as electron acceptors (oxidising agents) in deriving energy from the oxidation of organic matter. This typically occurs in the sequence (see Kirk 2004 for a full discussion): NO_3^- to N_2, Mn(IV) manganese to Mn(II), Fe(III) iron to Fe(II), and then SO_4^{2-} to S^{2-}. Subsequently organic matter is oxidised by

methanogenic bacteria to CO_2 and CH_4. This sequence is predicted by thermodynamics.

Concomitant with these redox reactions, there are important changes in the soil pH and alkalinity, and in the concentration of CO_2 dissolved in the soil solution. Protons are consumed in the various reduction reactions and the solution pH therefore tends to increase. Initially the pH may decrease following submergence because CO_2 formed in aerobic respiration escapes only very slowly, and it therefore accumulates. As CO_2 continues to accumulate during anaerobic respiration and fermentation, large partial pressures develop, typically in the range 5–20 kPa (Ponnamperuma 1972). The accumulation of CO_2 lowers the pH of alkaline soils and curbs the increase in pH of acid soils. As a result the pHs of all soils tend to converge following submergence in the range 6.5–7 (Ponnamperuma 1972).

As the partial pressure of CO_2 increases, the concentration of HCO_3^- in the soil solution increases and therefore the concentration of balancing cations in solution increases. The Mn^{2+} and especially Fe^{2+} ions formed in soil reduction displace exchangeable cations into solution. Also, the changes in pH cause changes in the charges of variable-charge clays and organic matter; thus the cation exchange capacity of acid soils tends to increase and that of alkaline soils tends to decrease.

The floodwater standing on the soil surface is usually sufficiently shallow, well mixed by wind and thermal gradients, and oxygenated by photosynthetic organisms that it is essentially aerobic (Roger 1996). However transport of O_2 into the underlying soil is too slow for more than a thin layer to be aerobic (Bouldin 1968; Patrick and Delaune 1972). In this aerobic layer the concentrations of reduced species are negligible, and CO_2 is the main end product of microbial respiration. In the underlying anaerobic soil, only a few millimetres away, the concentrations of Fe^{2+} and the various organic products of anaerobic respiration can be very large. Thus conditions change dramatically over a very short distance.

The most visible change associated with these processes is the reduction of the red and brown compounds of Fe(III) iron to blue-grey compounds of Fe(II) iron. Subsequent translocation of soluble Fe^{2+} to zones where O_2 enters the soil—such as at the soil surface or near plant roots—produces reddish-brown mottles of insoluble Fe(III) oxides. Likewise there may be movement and re-oxidation of Mn(II) forming black Mn(IV) compounds.

Though there are exceptions—notably acid sulphate soils—a feature of flooded rice soils is that they tend not to become acidic after continuous cultivation (Kirk 2004). In upland soils, acidification occurs chiefly because of the leaching of NO_3^- accompanied by base cations. But in general, little or no NO_3^- is leached from flooded soils, any NO_3^- entering the soil or formed in it being either absorbed by the crop or denitrified, so this process does not occur. Further, the anaerobic processes causing pH changes following flooding are reversed upon drainage and re-oxidation of the soil. Thus, unless there has been substantial movement of acid or base out of the soil during flooding, as generally there will not have been, the pH changes are reversed.

Nor do rice fields tend to become saline, except in arid or semi-arid areas (Greenland 1997). In land that is flooded for part of the year but drains naturally

after the floodwater recedes, accumulated salt is removed with the draining water and there is a natural renewal of the land. Percolation and lateral drainage at the start of the following rainy season, but before the land is re-flooded, also wash out accumulated salt, as in coastal areas.

4 Root Aeration

4.1 Root Anatomy

4.1.1 Aerenchyma and Porosity

Growth of vascular plants in flooded soils requires an efficient internal aeration system to deliver O_2 to submerged organs, with the additional benefit of venting CO_2 produced in the roots and soil to the atmosphere. Rice and other wetland plants contain extensive longitudinally interconnected gas-filled channels—aerenchyma—which provide a low-resistance pathway for gas-phase diffusion between shoots and roots (Barber et al. 1961; Armstrong 1979). Figure 3 shows aerenchyma in different root tissues of rice.

The aeration network is mainly based on aerenchyma, though in certain parts of the root system lacking lacunae, there may be regions with relatively high porosity due to cubic packing of cells; for example, between the end of lacunae and root apices, and in root laterals (Justin and Armstrong 1987). Maximum porosity with cubic packing if adjacent cells are perfectly spherical and only in 'point contact' is 21.5 %, compared to 9 % for hexagonal packing (Justin and Armstrong 1987). In practice these values are smaller in real root tissues because neighbouring cells have much more than just point contact. An example is given by Armstrong (1971) who found the porosity of the 1–2 cm zone behind the apex in rice roots grown in drained soil was 9 %, the roots not having aerenchyma and all the porosity being due to extracellular spaces between the cubically packed cells.

In a study of 41 wetland species in flooded compost, rice roots (cv. Norin 36) were eighth most porous (Justin and Armstrong 1987). At 35 % porosity rice was similar to other well-known wetland species such as *Spartina anglica* and *Juncus effusus*, but less porous than *Phragmites australis* (52 %) and *Juncus inflexus* (52 %). Porosity in the basal parts of rice roots reached approximately 45 % whereas values were approximately 10 % at 1 cm behind the apex where large aerenchyma channels are absent, but the cubical arrangement of cortical cells provides gas-filled spaces (Armstrong 1971). Moreover, rice genotypes differ in root porosity; Colmer (2003a) found a range of 29–41 % in 12 genotypes grown in stagnant deoxygenated agar. Armstrong (1971) found root porosity of rice was approximately 20 % in drained soil but 43 % in waterlogged soil. Similar findings were obtained for rice in aerated nutrient solution and in stagnant agar (Colmer 2003a). High porosity within roots in flooded anoxic soil is important because root

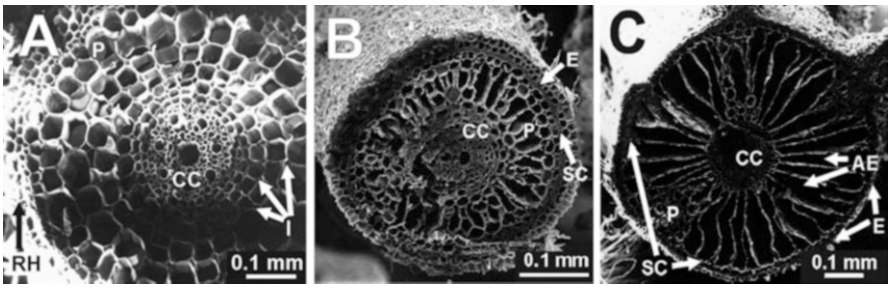

Fig. 3 Cross sections of primary rice roots (Butterbach-Bahl et al. 2000). (**a**) Radial section close to tip showing intercellular spaces (I), central cylinder (CC), and rhizodermis (RH). (**b**, **c**) Radial sections of younger (39 days) and older (72 days) basal parts showing exodermis (E), sclerenchymatous cylinder (SC), parenchyma of cortical cells (P), and aerenchyma (AE). Reproduced by permission of the authors

growth depends on internal diffusion of O_2 to the apex (Armstrong and Webb 1985). The capacity for this supply determines maximum rooting depth and together with a barrier in the outer cell layers of roots (Sect. 4.1.2) influences radial O_2 loss and rhizosphere biogeochemical processes (Sect. 6).

Root aerenchyma are connected to lacunae at the shoot base, through which O_2 enters from the atmosphere above. In a study in which porosity was measured in leaves, sheath bases, and roots of rice (cv. Calrose), Colmer and Pedersen (2008) found porosity was 26 ± 2 % (v/v) in roots, 40 ± 4 % in sheaths (basal 100 mm), and 29 ± 2 % in leaf blades including the mid-rib, with the largest lacunae in leaf blades being in the mid-rib. The importance of the shoot as the O_2 source for the roots in an O_2-free medium was demonstrated in experiments in which O_2 was manipulated (either exogenously by gas mixtures or endogenously via light/dark periods) and dynamic tissue O_2 concentrations monitored using O_2 microelectrodes and root-sleeving electrodes. A low-resistance pathway for O_2 movement was present from leaf mid-rib to sheath base to roots (Colmer and Pedersen 2008). Oxygen movement along submerged shoots can also occur within surface gas films (see Colmer et al. 2013). For rice in shallow water, the very high O_2 concentrations in the floodwater (0.31–0.62 mM—Sect. 3.1) may increase the O_2 in the adjoining shoot tissue well above ambient, in which case the source of O_2 for diffusion into the roots would be greater than ambient.

Rice genotypes also differ in the extent of shoot aerenchyma (e.g. sheaths, Parlanti et al. 2011; stems, Steffens et al. 2011) and there are differences between growth stages and development patterns. Some aerenchyma formation in shoots is ‘constitutive’, as it is in roots (Steffens et al. 2011). Ethylene signalling has been implicated in the flood-induced enhancement of aerenchyma formation in both root (Justin and Armstrong 1991; Colmer et al. 2006) and shoot tissues, with involvement also of H_2O_2 in the programmed cell death that forms the lacunae in shoots (Parlanti et al. 2011; Steffens et al. 2011).

That transport of O_2 within aerenchyma is by diffusion was first demonstrated by van Raalte (1940), who measured concentration profiles of O_2 from rice shoots to roots in semi-stagnant solution using micro-manometers. Oxygen concentrations (means of two rice cultivars) were 12.5 % near the base of the root, 6.6 % in the middle, and 4 % at the tip. Subsequently, Armstrong and co-workers have elucidated the processes of internal aeration in plant roots by use of mathematical models integrated with experimental data on axial and radial O_2 profiles in maize roots and the rhizosphere, obtained with root-sleeving electrodes and micro-electrodes (Armstrong 1979; Armstrong and Beckett 1987; Gibbs et al. 1998; Darwent et al. 2003). These studies demonstrate the importance of aerenchyma in providing a low-resistance pathway for diffusion of O_2 and other gases within the roots.

In addition to their large gas-filled porosity, roots of rice also contain a barrier to radial O_2 loss (ROL) in the basal zones (Sect. 4.1.2). The barrier restricts O_2 loss from the root, and thereby permits a greater length of root to be aerated by diffusion, resulting in a higher apical O_2 concentration and an aerobic rhizosphere around the root tip (Armstrong 1979). In contrast to the data available on internal aeration and anoxia tolerance in rice, there is little information on metabolism as related to spatial and temporal differences in O_2 supply. In spite of the ventilation system, the various root tissues will experience a range of O_2 concentrations, as a function of their position along the diffusion path, and the external sink for O_2.

4.1.2 The Barrier to O_2 Loss

The barrier to radial O_2 loss (ROL) from the root is located in the outer cell layers of the main axis of the roots. This has been shown for various wetland plants (Armstrong 1979; Colmer 2003a) and rice (Armstrong 1971; Colmer 2003b; Shiono et al. 2011). In the relatively few rice varieties investigated so far, the barrier starts at 1–1.5 cm behind the root tip and has a very low permeability to O_2; ROL from the main axis in the basal regions was more than two orders of magnitude less than at the root tip—despite the higher expected internal concentrations in the basal zones (Armstrong 1971; Colmer 2003b). In roots of rice, the barrier to ROL is inducible, forming in stagnant or waterlogged conditions, but not (or only weakly) in aerated conditions (Colmer et al. 1998, 2006; Colmer 2003a; Shiono et al. 2011). Many wetland species have a ROL barrier in their roots; some species constitutively form such ROL barriers even in aerated conditions whereas in others it is induced by growth conditions (Colmer 2003b), but there are also notable exceptions such as *Rumex palustris*. The ROL barrier in *R. palustris* is rather weak by comparison with rice, as shown by the similar rates of ROL between root base and root tip (Visser et al. 2000). With no barrier at all, diffusion models predict a hyperbolic decrease in O_2 concentration within the aerenchyma towards the root tip, and a similar profile for ROL to the medium (Armstrong 1979).

One advantage of the barrier to ROL is the potential for a greater rooting depth (Armstrong 1979). The importance of the barrier becomes less as root porosity

increases, unless roots are in highly reduced substrates, which contain large concentrations of organic or inorganic toxins produced by anaerobic bacteria. Having longer roots presumably enables exploration of a greater volume of soil from which nutrients can be absorbed. However, the acclimative advantage of having a barrier to ROL may depend on soil type, with the greatest importance presumably being in highly reducing soils (Sect. 5.4). In a study with 12 genotypes of rice in stagnant agar, the barrier did not form in two of the seven upland genotypes included in the study (Colmer 2003a). So, genotypic variation for this trait within rice should allow further study of the importance of the barrier in soils with different redox potentials.

4.2 The Root O_2 Budget

4.2.1 Oxygen Loss from Lateral Roots

Short, fine lateral roots account for a large proportion of the absorbing root surface in rice (Matsuo and Hoshikawa 1993) and these roots leak more O_2 on a per plant basis than the adjacent primary root. Contributory factors are that these laterals have no impermeable wall layers (except under exceptional circumstances—Armstrong and Armstrong 2005) and they have a large surface area to volume ratio (Armstrong et al. 1990; Kirk 2003). Evidence for preferential loss of O_2 from laterals includes measurements of Fe oxide coatings on roots placed in deoxygenated agar containing Fe(II) (Trolldenier 1988); changes in redox potential as roots grew across rows of platinum electrodes in anaerobic soil (Flessa and Fischer 1993); and the abundance of methane oxidising bacteria, which are obligate aerobes, along rice lateral roots in anaerobic soil (Gilbert et al. 1998).

Kirk (2003) developed a simple model of root aeration in rice in relation to root architecture and concluded that the basic architecture of current rice genotypes provides the best compromise between the need for internal aeration and the need for the largest possible absorbing surface per unit root mass. This architecture consists of coarse, aerenchymatous primary roots with gas-impermeable walls conducting O_2 to short, fine, gas-permeable laterals.

As in rice, some other wetland species develop prolific laterals near the root base (i.e. just below the shoot–root junction) which conduct substantial amounts of O_2 to the waterlogged soil. In *Phragmites australis*, for example, these basal laterals conduct 70–100 times more O_2 to the soil than the main axes (Armstrong et al. 1990). In *Phragmites*, redox potentials near the root were between 200 and 600 mV, while the bulk soil was −280 mV (Armstrong et al. 1990). The O_2 movement to the rhizosphere from these shallow laterals, emerging from near the root base, as opposed to deeper laterals, would mitigate drops in the O_2 gradient within the aerenchyma of the main axis, in view of the short distance between the O_2 source and the soil sink (Armstrong 1979). Hence substantial amounts of O_2 can be released to the soil without compromising aeration of deeper root portions.

Nonetheless, in an emergent aquatic plant, *Eleocharis sphacelata*, models assuming a 30 cm long root and 6 kPa at the root–rhizome junction indicated that ROL from laterals could lower cortical O_2 concentrations at 10 cm from the rhizome–root junction from approximately 5.5 down to 1.5 kPa (Sorrell 1994).

Experiments in sterile versus non-sterile media show that the degree of oxygenation can vary substantially with the presence of bacteria: Hojberg and Sorensen (1993) found the O_2 concentration at the surface of a barley root was 9 kPa in an otherwise O_2-free sterile gel medium but only 2–4 kPa when non-sterilised soil extract had been added to this medium. In this latter medium, the rhizosphere respired 30–60 times faster than the bulk medium (Hojberg and Sorensen 1993).

4.2.2 Oxygen Consumption in Root Tissues

A long-standing question is the effect of different volumes of aerenchyma on the relative apportioning of O_2 to the root tissues and the rhizosphere. One factor determining O_2 concentration in the root tip is respiratory consumption by the root tissue along the pathway. It has been suggested that degradation of the cortex to form aerenchyma would not only decrease the physical resistance for gas-phase diffusion but also have the added benefit of reducing the amount of respiratory tissue (Williams and Barber 1961). However, using an electrical analogue and data for wetland and non-wetland plant roots, Armstrong (1979) concluded that the role in the decreased diffusional resistance for O_2 provision via the aerenchyma was dominant.

Armstrong (1979) found that the length of non-wetland roots reached 6 cm and that of rice roots 10 cm under his standard conditions. The O_2 concentrations at 6 cm from the root–shoot junction in rice were 15.2 kPa, decreasing to 12.0 kPa when the analogue assumed that both respiratory demand and radial O_2 loss were as high as in a dryland-type root. By contrast, for a non-aerenchymatous dryland-type root, O_2 at 6 cm from the root–shoot junction only increased from 2.3 to 5 kPa when the model assumed low radial O_2 loss and a typical respiratory demand for a wetland plant, but with no aerenchyma.

Armstrong and Beckett (1987) extended the modelling of O_2 transport and consumption within roots to include differences between tissue cylinders in the radial direction. This indicated that the various tissues across roots differ in O_2 availability, and these differences become substantial in roots receiving a limited O_2 supply, particularly in the most distal parts of the roots. These ‘multi-shell models’ predict energy deficits developing in two crucial tissues, which both have a very low porosity and a high metabolic activity. These tissues are the root tip, which is at the end of the longitudinal path of O_2 from the shoot base, and the stele which, at least in maize, may display anaerobic catabolism even earlier than the root tip (Armstrong and Beckett 1987). These observations led to the hypothesis that, in an anoxic stele, loading of nutrients into the xylem is inhibited, leading to a substantial decrease in nutrient flow to the shoots (Gibbs et al. 1998; Colmer and Greenway

2011). For a vigorously growing crop like rice, such inhibition of nutrient transport to the shoots might be quite detrimental (but see Sect. 5.2).

4.2.3 Mechanism of Gas Movement: Diffusion or Pressure-Driven Mass Flow?

Some wetland species have an aeration potential far in excess of that provided by diffusion: bulk through-flows of "air", described by Dacey (1980) as 'internal winds', are driven by real pressure differences from emergent living shoots/leaves/leaf sheaths into submerged rhizomes and the 'exhaust' gases escape from other emergent parts (Armstrong et al. 1991, 1992; Brix et al. 1992). Although *Oryza sativa* does not have rhizomes, the tillers are connected and it is conceivable that bulk gas flows could be generated between tillers having differential potentials for pressure generation. However, there is no evidence for this and in the following three truly rhizomatous *Oryza* species, no pressurisation or through-flow has been detected (Armstrong and Armstrong, unpublished data): *O. rhizomatous* (from Sri Lanka; IRGC Accession no. 105448), *O. australiensis* (IRGC Accession no. 103303), and *O. longistaminata* (from West Africa). It should be tested whether there is through-flow in any of the other wild *Oryza* species that possess true rhizomes.

An important controversy has been whether in rice there is a substantial contribution of mass flow to O_2 provision from the atmosphere via gas films on the submerged parts of the shoots (suggested by Raskin and Kende (1983), based on short-term observations). However, mass flow along partially submerged rice shoots will not substantially enhance total O_2 supply, because in the absence of a through-flow pathway, such mass flow merely replaces rather than augments flow by diffusion (Beckett et al. 1988).

4.2.4 Testing Models of Root Aeration

Experimental support for models of root aeration is equivocal. Predicted rooting depths in anoxic media as determined by gas-filled porosity influencing O_2 movement to root tips are broadly in agreement with experimental data. For example, Thomson et al. (1992) found good agreement of actual root lengths with those predicted by Armstrong's (1979) 'root length model', with the length of rice roots with 42 % porosity reaching 24 cm, compared to wheat roots with 22 % porosity reaching only 14 cm. These calculations did not allow for ROL, which would be much greater in wheat than in rice; however, the earlier referred to model by Armstrong (1979) showed in non-wetland roots the O_2 concentration at 6 cm from the root–shoot junction would only rise from 2.3 to 5 kPa when a ROL value typical of rice was used, so its effect on maximum root length might be rather hard to pick up experimentally.

The adequacy of the internal aeration system for roots of rice can be tested by O_2 measurements in root tissues such as the stele and particularly towards the tips near

the end of the longest diffusion pathway. Additional data on soil oxygenation especially near lateral roots are also needed. Experience with roots of maize and *P. australis* has shown that, though valuable, such measurements cannot easily be used to decide whether O_2 concentrations are adequate for aerobic catabolism. So, one way forward would be to dovetail these O_2 measurements with checks on metabolism and root functioning in energy-dependent ion transport. One powerful approach would be the concurrent measurements of O_2 concentration profiles and O_2 fluxes along with ion fluxes for roots under defined conditions, using micro-electrodes, as Pang et al. (2006) have done with roots of barley. (Note: in applying such methods, interpreting O_2 fluxes into roots when the shoot system is also supplying O_2 can be very difficult and can lead to erroneous conclusions.) There are now also CO_2 micro-electrodes (e.g. Anjos and Hahn 2008), but these have not yet been used in studies on rice or other plants.

One key question is whether the earlier mentioned O_2 of 34–68 kPa (i.e. approximately two to three times higher than ambient) in the shallow floodwater of rice paddies during the day (Sect. 3.1) would substantially influence the O_2 regimes of the root. Similarly, the near zero O_2 concentrations at night may lower the O_2 concentration at the root–shoot junction, and thus also within roots. If so, there might be diurnal fluctuations in root elongation and the thickness of the oxygenated rhizosphere. This complex situation needs to be explored by modelling dovetailed with experiments (e.g. with micro-electrodes in the field, as recently achieved with rice when completely submerged (Winkel et al. 2013)).

4.3 The Root CO_2 Budget

The aeration network also plays an important role in controlling CO_2 partial pressures in roots in flooded soil, despite the high CO_2 levels that at times develop in the surrounding soil (Greenway et al. 2006). There are few experimental data on CO_2 partial pressures within roots in flooded soils. However Greenway et al. (2006) modelled CO_2 concentrations as being dependent on the removal of CO_2 through root aerenchyma for given rates of CO_2 production in root tissues and entry from the rhizosphere. They calculated that in 10 cm long roots with 0.6 mm diameter, 35 % porosity, and 40 kPa CO_2 in the soil, the CO_2 partial pressure in the root would not rise above approximately 13 kPa because there would be continuous venting to the atmosphere. The model also assumed that gas exchange between soil and root was restricted to the 10 mm tip. Greater CO_2 would occur with narrower and/or longer roots (Greenway et al. 2006).

Another approach to predict CO_2 partial pressures in roots in flooded soils is based on observed O_2 partial pressures in the roots, by making the simplifying assumption that the only way to ventilate the CO_2 produced by the plant roots is via the aerenchyma to the overlying atmosphere. It is reasonable to assume that the CO_2 production at least equals the O_2 consumption, i.e. that the respiratory quotient (CO_2/O_2) is one. Now, if the root contains 10 kPa O_2, which is a rather high value

for much of the roots of rice in anoxic soil, then the CO_2 partial pressure would also be about 10 kPa, because equal amounts of O_2 and CO_2 would have to diffuse through the aerenchyma, albeit in opposite directions, and so they would require similar concentration gradients. The CO_2 pressure in the root may be even greater if there is ethanol fermentation (and hence additional CO_2 release). The restriction that ventilation would be only via the aerenchyma is likely for roots in many rice soils, since 10 kPa CO_2 in these flooded soils is a modest value (Ponnaperuma 1972).

Even the modest CO_2 partial pressure of 10 kPa may have profound consequences for the root tissues. Taking the cytoplasmic pH to be 7.5, there would be 75–90 mM HCO_3^- in the cytoplasm (Greenway et al. 2006). This HCO_3^- load could be coped with in three ways: (1) decarboxylation of endogenous organic acids, providing cations to balance the HCO_3^-, but to a maximum of the prevailing concentration of organic anions, usually approximately 35 mM (Greenway et al. 2006); (2) increases in K^+ concentration in the cytoplasm, with the risk that the K^+ concentration becomes excessive; and (3) allowing a decrease in pH of the cytoplasm, which would greatly decrease the HCO_3^-equilibrium concentration (a decrease of 45 % for a decrease in pH of only 7.5 down to 7.3).

The HCO_3^- load may be even more acute in root tips, which can grow with internal O_2 lower than 1 kPa (measurements in de-oxygenated agar solution, Armstrong and Webb 1985). So, depending on the CO_2 partial pressure in the soil and still assuming a respiratory quotient of one, internal CO_2 might rise to approximately 20 kPa. Thus, we suggest that at least in some rice soils, it is hard to predict whether the roots would suffer from O_2 deficiency, excess CO_2, or both. As one example, soils high in organic matter develop large CO_2 concentrations after flooding and that would tend to increase the CO_2 partial pressure within roots (Greenway et al. 2006). The opposite would be the case for soils low in CO_2 because outward diffusion could mitigate the internal rise in root CO_2 caused by root respiration and perhaps ethanolic fermentation. However exit of CO_2 would also depend on the permeability of the outer cell layers of the roots which in rice contain a gas-diffusion barrier.

5 Root Function

5.1 Root Elongation

The limiting factor in root elongation of completely submerged rice is probably the O_2 concentrations in the root apex. Evidence for this includes the very quick cessation of elongation when lights are switched off for submerged rice, when ROL from just behind the root tip rapidly dropped to zero (Waters et al. 1989). When subsequently light was again provided, both ROL and root extension resumed rapidly. Consistently, in rice, manipulation of O_2 around the shoots

showed that root elongation slowed when O_2 at the root tip dropped to 1 kPa and only ceased altogether when O_2 at the tip dropped to 0 kPa (Armstrong and Webb 1985). The simplest explanation for the cessation of elongation is that the root tips come under a severe energy crisis, so the limited energy produced is spent on maintenance, i.e. directed towards survival, rather than to growth.

Further experimentation with rice, using manipulations of O_2 around the shoots, would be revealing, both for more detailed testing of the responses of extension and particularly of nutrient uptake, which as far as we know has not been done for any species.

5.2 Metabolism

Presently, there are only very few data available on metabolism in different parts of roots subjected to O_2 deficiency, and as far as we know none of these is for rice. Hence we discuss first the case of maize roots, for which some data are available, and then consider under what conditions similar events may occur in rice roots.

Metabolic evidence for the effect of the O_2 gradient from the root–shoot junction to the root tip of intact maize plants with roots in an anoxic medium was presented decades ago (Drew et al. 1985). The ATP/ADP ratios at 0–100 mm from the root–shoot junction were about four in both aerenchymatous and non-aerenchymatous roots, while these ratios in the root tip, at 155–200 mm from the shoot–root junction, were approximately 1.7 and 0.71 in aerenchymatous and non-aerenchymatous roots, respectively (Drew et al. 1985). Further support for O_2 deficiency in the root tip, even though at least the cortex in most of the root still receives plenty of O_2, is sketchy. However when whole maize roots were exposed to 0.6 mM O_2 in the nutrient solution, alanine in the tip increased from 4 to 33 mM (Gibbs et al. 1998). Alanine is a reasonable indicator of O_2 deficiency as it accumulates to high concentrations in anoxic rice coleoptiles and several other tissues (Gibbs and Greenway 2003).

Turning to possible O_2 deficiency in the stele, in maize at locations where the cortex was at 4.3 kPa O_2, O_2 was below detection levels in most of the stele (Gibbs et al. 1998). Consistently, Cl^- transport into the xylem was 50 % lower than in aerated roots (Gibbs et al. 1998). These observations led to the hypothesis that anoxia in the stele inhibits the transporters which load ions into the xylem vessels (Colmer and Greenway 2011). Thus, after the initial energy-dependent uptake by the cells of the epidermis and the cortex, which still receive adequate O_2, the subsequent transport of ions to the xylem would become dependent on diffusion and/or bulk flow as water moves to the xylem (Colmer and Greenway 2011).

Radial gradients of O_2 concentration between cortex and stele were also present for aerenchymatous maize roots (Darwent et al. 2003). Further, early stelar anoxia was suggested for the emergent aquatic species *Eleocharis sphacelata*, since the roots ceased elongating, though there should have been high enough O_2 pressures at the root tip. Interestingly, this paper suggested that stelar anoxia was more

widespread when the endodermis was lignified (Sorrell 1994). Oxygen deficiency in the stele of maize was further supported by metabolic measurements, such as concentrations of ethanol and the higher active state of the key enzyme of ethanol formation pyruvate decarboxylase in the stele than in the cortex (Thomson and Greenway 1991).

Overall these data suggest a large effect of root anaerobiosis on nutrient transport to the shoots, so it is worth exploring to what extent the events in maize occur in rice. No data on O_2 concentrations in stele versus cortex are available for rice. However anoxia in the stele of rice roots is less likely than in maize roots because rice has a narrow stele, i.e. a high ratio of absorbing interface between stele and cortex to volume of respiring tissue of the stele. The higher the ratio the less likely the stele will suffer O_2 deficiency. In the following, we have calculated this ratio from the areas given in publications (NB circumference $\times$ height / volume gives units $mm^2/mm^3 = mm^{-1}$). Data of McDonald et al. (2002) for adventitious rice roots give a ratio of 19 mm^{-1}, both under aerated and stagnant conditions, whereas data of Gibbs et al. (1998) for primary maize roots give a ratio of 6.1 mm^{-1}. In general, wetland plants often have narrow steles: in the data of McDonald et al. (2002) for adventitious roots, seven out of ten wetland plants had ratios of 10 mm^{-1} or greater and all three non-wetland plants tested had ratios of 5.2–6.1 mm^{-1}. So, impairment of ion transport to the xylem in rice is less likely than in maize. From model calculations (Armstrong and Beckett 1987), anaerobiosis in the stele of rice roots would only occur when O_2 availability becomes very restricted, either when the soil is very reducing and therefore becomes a strong competitive sink for O_2, or in distal root parts (e.g. stele just behind root apices and also the apices themselves).

Further exploration of these issues will not be as easy as in maize, since in that species the stele can be stripped readily from the cortex (Gibbs et al. 1998; Thomson and Greenway 1991). Apart from measuring ion fluxes to the shoot, suitable methods would be using micro-electrodes for membrane potentials, cytoplasm pH, and ion fluxes in the roots. Advantages of micro-electrodes are that the measurements can be on very specific tissues and will often respond very rapidly to a change in conditions. For example, in root hairs of *Medicago sativa* the imposition of anoxia depressed the pH of the cytoplasm by 0.5 within 2 min and shifted the plasma membrane potential from −160 to −125 mV (Felle 1996). Complementary measurements may be on key anaerobic metabolites and/or changes in gene expression, which are sensitive indicators of an energy deficit. Use of laser microdissection followed by analysis of different tissues using, for example microarrays (e.g. Rajhi et al. 2011) and other micro-assays, would also be revealing.

5.3 Nutrient Uptake

It has been speculated that the barrier to radial O_2 loss may impede nutrient uptake (Armstrong 1979; Colmer 2003a). Experiments with rice roots indicate that the ROL barrier itself, however, does not substantially impede nutrient uptake.

Rubinigg et al. (2002) grew rice in stagnant agar to induce the barrier and then measured uptake under aerobic conditions to exclude direct inhibitory effects of O_2 deficit on uptake. They found that NO_3^- net influx from 0.1 mM NO_3^- in the external medium, measured using a NO_3^--specific micro-electrode, was 3–5 μmol g^{-1} fresh weight h^{-1} in roots with and without the barrier. In the former the 20–50 mm section had at most 6 % of the ROL of roots without the barrier. So, although nutrient uptake under anoxia was not tested, this experiment shows that at least if the degree of barrier formation in soil-grown roots was similar to that in the deoxygenated agar, then it would be no hindrance to uptake in flooded soil that is drained and re-aerated, as in rainfed rice.

An impermeable barrier can still have some 'passage areas' ('windows'), as observed in *Phragmites* when O_2 at the root surface was 2–2.5 kPa compared to the main adjoining areas which were close to zero O_2 (Armstrong et al. 2000). Armstrong et al. (2000) postulated that the windows in the barriers might facilitate emergence of laterals or nutrient uptake, or both. The former might be important when the barrier is strong. However, the suggestion on the facilitation of transport has the problem that the fraction of these passage areas relative to the total root surface area would have to be either substantial, in which case the function of the barrier to prevent O_2 loss would be compromised, or the nutrient uptake across the passage areas would have to be many fold faster than that usually encountered for root cells. However, it is possible that the cells of the passage areas have an unusually high uptake capacity, as, for example, in salt glands and in cells loading ions into the xylem.

Having excluded the possible impedance to nutrient influx by the barrier per se, the intriguing question remains whether in roots in flooded soils the epidermal cells outside the barrier to ROL can absorb nutrients even though these would be exposed to very low O_2. Alternatively, diminished rates of absorption by roots with the barrier may be compensated for by greater uptake in zones without the barrier, and by lateral roots. Calculations of the extent to which N uptake by rice in flooded soil is limited by root uptake properties versus transport through the soil to root surfaces suggest that it is the fine laterals that are responsible for the bulk of the uptake (Kirk and Solivas 1997).

Colmer (2003a) found dry weights of shoots and roots in nine of 11 rice cultivars were the same or 20 % greater in waterlogged than in drained soil. Thus, either the root sections with the barrier to radial O_2 movement took part in the required nutrient uptake, or other parts of the root system compensated for decreased nutrient intake in the sections with the barrier or restricted rooting depth or both. Similarly, net N uptake rates did not differ between roots of rice grown in stagnant deoxygenated agar or in aerated, nutrient solution (Rubinigg et al. 2002).

Kronzucker et al. (1998a) studied the effects of hypoxia on NH_4^+ fluxes in rice roots in nutrient culture using a ^{13} N tracer technique. They found little impairment of uptake capacity over 7 days of exposure to O_2 concentrations equivalent to 15 % of air saturation. Half-lives for NH_4^+ exchange with sub-cellular compartments, cytoplasmic NH_4^+ concentrations, and efflux (as percentage of influx) were unaffected by hypoxia, but there were differences in the relative amounts of N allocated

to assimilation and the vacuole versus translocation to the shoot. Measurements of influx with NH_4^+ concentrations in the high-affinity transporter range (2.5–500 μM) and varying O_2 saturation showed that the maximum influx (V_{max}) varied with the degree of hypoxia, but the affinity for NH_4^+ (K_m) was unchanged.

Most experiments testing responses to anoxic media have been made at high nutrient levels, but, as shown below, the outcome at lower levels may be different. Testing response to phosphate in stagnant solution (0.1 % agar), Insalud et al. (2006) found that relative growth rates were depressed at 1.6 μM, but not at 200 μM. This growth reduction was associated with a 90 % lower P uptake at 1.6 μM Pi, but only a 50 % reduction at 200 μM P_i in stagnant compared to turbulent solution. Evidently the high-affinity P transporters that operate at lower P concentration were more affected by hypoxia. Similarly, Kotula et al. (2009a) found that rice in stagnant agar grew poorly compared to aerated solutions (photographic evidence only). This inhibitory response contrasted with the better growth and nutrient uptake in stagnant agar found by Rubinigg et al. (2002) and Colmer et al. (2006). The simplest explanation is the low nutrient concentrations used: P_i, 0.05 mM and 0.15 mM N in Kotula et al. (2009a), but 0.2 mM P and 5 mM N (7:1, NO_3^-:NH_4^+) in Rubinigg et al. (2002) and Colmer et al. (2006). Similarly, K and N concentrations were in the 0.15 mM range in Kotula et al. but 1 mM or higher in Colmer et al. (2006). In stagnant agar, limitations to nutrient uptake may be due to either O_2 deficiency or impeded nutrient diffusion (Wiengweera et al. 1997). The relative importance of these factors can be resolved by increasing the O_2 concentrations around the shoots and so elevating the O_2 concentrations in the root as has been used for root elongation by Wiengweera and Greenway (2004), as well as by nutrient dose–response experiments.

Further elucidation of mechanisms of root aeration, nutrient acquisition, and growth under less optimum conditions, for example under low levels of mineral nutrition, should be a focus of future research. In addition, understanding of the importance of root growth and proliferation of laterals to access nutrients of low solubility such as phosphate (Sect. 6.2.2), and implications of root functioning in soils with fluctuating water levels (drained–flooded–drained cycles), will be important avenues for understanding of limitations for rice productivity in these conditions.

5.4 Exclusion of Toxins

The second important function of the barrier to radial O_2 loss might be prevention of influx of toxic elements that are present in soil of low redox potential. Armstrong and Armstrong (2005) found that increased root cell wall suberisation, induced by high sulphide concentrations in stagnant nutrient culture, caused decreased absorption of Fe^{2+}. Kotula et al. (2009b) found the barrier to ROL greatly decreased the diffusion of Cu^{2+} through the apoplast. These results do not contradict unimpaired NO_3^- net uptake in the presence of the barrier (Sect. 5.3) because the uptake would

be mainly by the epidermis, with subsequent flow through the symplast. Furthermore, energy-dependent NO_3^- uptake would have a high ratio of flux to external concentration compared with Fe^{2+} uptake along a free energy gradient.

However, where this function of the barrier to block toxin entry is important (i.e. in highly reduced soil), the uptake of essential elements by the zones with the barrier may also decline markedly, because the metabolism of the epidermal cells will be inhibited even if the concentration of the soil toxin is not lethal to the epidermal cells. The lignification/suberisation of the outer cell layers can become much more intense in the presence of reduced-soil toxins, to the extent that the physical barrier prevents emergence of laterals and they grow upwards within the parent adventitious root (Armstrong and Armstrong 2005). This would result in a smaller root surface area for nutrient absorption.

5.5 Hydraulic Conductivity

Colmer and Greenway (2011) have recently reviewed the few data on this issue. The evidence is inconclusive but one positive finding was that in rice roots, the large volume of aerenchyma is unlikely to add to the hydraulic resistance since water flowed along the rim of the living cells of the strands connecting the outer root with the stele (Miyamoto et al. 2001).

Kotula et al. (2009a) have shown that the permeability of the outer cell layers in roots of rice to water was 10-fold smaller than that for O_2. However, these authors emphasised that in transpiring plants this slow diffusion is irrelevant, since the hydraulic conductivity was 600–1,400 times greater than the diffusive conductivity. These data were for roots of aerated plants, so it remains important to establish the effect of the barrier to O_2 loss on hydraulic conductivity.

6 The Rhizosphere

6.1 Root-Induced Changes in the Soil

As a result of O_2 loss from the root, and other root-induced changes, conditions in the rhizosphere 1–2 mm from the root surface can be greatly different from those in the anoxic bulk soil. This has important consequences for the concentrations and uptake of nutrients and toxins. The main processes are as follows (Fig. 4; see Kirk 2004 for a fuller discussion).

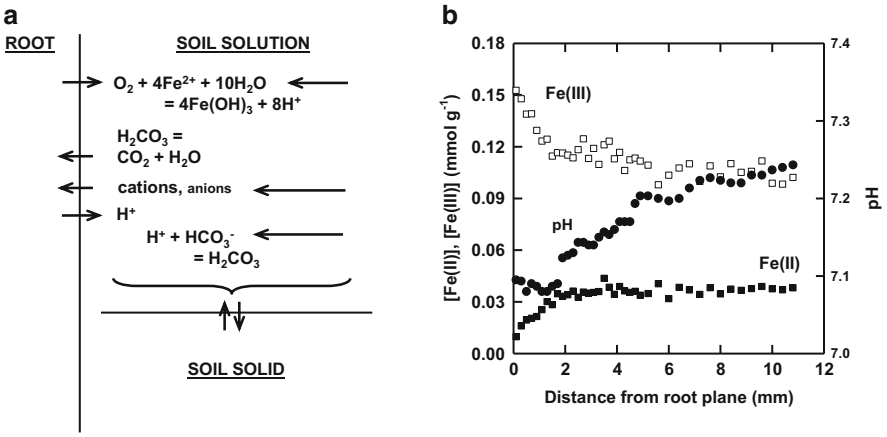

Fig. 4 (**a**) Processes in the rice rhizosphere. (**b**) Profiles of ferrous and ferric iron and pH in blocks of a reduced soil in contact with a planar layer of rice roots for 12 days (Kirk and Bajita 1995)

6.1.1 Oxygenation

As discussed in Sect. 4, some of the O_2 diffusing down through the aerenchyma leaks out into the soil where it is consumed in biotic and abiotic processes. Mobile inorganic reductants in the soil are oxidised, particularly Fe^{2+} which is precipitated as $Fe(OH)_3$ on or near the root. As a result the concentration of Fe^{2+} near the root falls and more Fe^{2+} diffuses in from the bulk soil. This is then oxidised resulting in a zone of $Fe(OH)_3$ accumulation near the root. Hence reddish-brown deposits of ferric oxide are frequently observed on the older parts of rice roots.

The flux of O_2 across a particular portion of the root depends not only on the rate of O_2 transport through the root (Sect. 4.1) but also on the strength of the sink presented by the external medium. In soil, the sink strength depends on the rate of O_2 diffusion into the soil, its rate of consumption by microbes and reaction with mobile reductants such as Fe^{2+}, and the rate of Fe^{2+} diffusion towards the oxidation zone (Bouldin 1968). There are also differences along the root length, and between primary roots and laterals (Sect. 4.2.1). As a root grows through a portion of soil, a zone of Fe^{2+} depletion arises where oxidation is intense in the region of the root tip, but is rapidly filled in when the O_2 supply decreases as the less permeable parts of the root grow past. Re-reduction of Fe(III) is slow compared with oxidation of Fe(II). Hence the root tips are generally white and free of ferric oxide deposits, whereas the older parts are coloured orange brown.

Measured fluxes of O_2 from the roots of rice and other wetland species vary greatly, depending on the method used and experimental conditions. Sorrell and Armstrong (1994) discuss the difficulties in making these measurements. Methods include the use of polarographic electrodes to measure fluxes from sections of individual roots (Armstrong 1979); measurements of O_2 release into continuously

replenished, de-oxygenated solutions bathing whole root systems (Bedford et al. 1991; Kirk and Du 1997); measurements of Fe(II) oxidation in the rhizosphere near planar layers of roots in reduced soils (Begg et al. 1994; Kirk and Bajita 1995); and use of O_2 micro-electrodes to measure gradients of O_2 concentration near roots growing in field soils (Revsbech et al. 1999). The differences between methods reflect the various complexities listed above, and particularly the importance of differences between different parts of the root and root system, and differences over time. From the point of view of processes in the rhizosphere, the fluxes of O_2 from root tips and laterals are of a similar magnitude to fluxes of nutrient ions into roots—of the order of a few nmol dm^{-2} root s^{-1}—and, given the effects of radial geometry, these fluxes are sufficient to greatly alter the soil chemistry and biology close to root surfaces.

6.1.2 Acidification

The oxidation of inorganic reductants generates protons:

$$4Fe^{2+} + O_2 + 10H_2O = 4Fe(OH)_3 + 8H^+$$

so the pH in the oxidation zone tends to fall. Further, because the main form of plant-available N in anaerobic soil is NH_4^+, the root absorbs an excess of cations over anions. Consequently H^+ is released by the root to maintain electrical neutrality, further decreasing the soil pH. Note that if N is taken up as NO_3^- as a result of nitrification of NH_4^+ in the rhizosphere, the net acid–base change is the same because, although the root exports 2 mol less H^+ for each mol of NO_3^- replacing a mol of NH_4^+, 2 mol of H^+ are formed in the nitrification of each mol of NH_4^+. Note also that Si, which is taken up in large quantities by rice plants, crosses the root as the uncharged H_4SiO_4 molecule ($pK_1 = 9.46$ at 25 °C).

The net effects of these processes will depend on the rate of H^+ generation versus the rate at which H^+ moves away by acid–base transfer. In flooded soils, acid–base transfer tends to be fast, both because soil diffusion coefficients increase with water content and because the high concentration of dissolved CO_2 results in a high concentration of the acid–base pair H_2CO_3–HCO_3^-. Hence protons are transferred by reaction with HCO_3^- and diffusion of more HCO_3^- into the acidification zone from the bulk soil. Modelling and experimental results (Begg et al. 1994; Kirk and Bajita 1995) show that over realistic ranges of conditions the rice rhizosphere will be acidified by up to 0.5 pH units as a result of these processes, and in certain circumstances by more than 1 pH unit.

6.1.3 CO_2 Uptake

Because very large concentrations of dissolved CO_2 develop in flooded soil (Sect. 3.2.2), in spite of root respiration the CO_2 pressure outside the root may be greater than that inside it, resulting in a flow of CO_2 from the soil to the atmosphere through the aerenchyma. The consequences for root physiology are discussed in Sect. 4.3. Net removal of CO_2 by the root decreases the concentration of the acid H_2CO_3 near the root, and this may offset the acidity produced in oxidation and excess cation uptake (Begg et al. 2004).

6.2 Consequences for Nutrients and Toxins

6.2.1 Ammonium Versus Nitrate Nutrition

The principal form of plant-available N in chemically reduced, flooded soils is NH_4^+, formed from the mineralisation of organic matter or from mineral fertilisers. This contrasts with well-aerated soils, where the principal form is generally NO_3^-. Because NH_4^+ is adsorbed on soil surface, both as a freely exchangeable cation and in more tightly held forms within clay lattices, much smaller concentrations develop in the soil solution than of NO_3^- in well-aerated soils. Rates of transport to roots by mass flow and diffusion are correspondingly slower and potentially limit uptake. Kirk and Solivas (1997) examined this question with a model and experiments, and concluded that in well-puddled flooded soil rates of transport will generally not limit NH_4^+ uptake rates for typical rice rooting densities.

Potentially more important are the consequences of NH_4^+ nutrition for plant physiology. Plant growth and yield are generally better when plants absorb their N as a mixture of NH_4^+ and NO_3^- rather than as either on its own (Hawkesford et al. 2012). The reasons are not fully understood, but may involve the need to provide carbon skeletons for NH_4^+ assimilation in roots, compared with NO_3^- assimilation in shoots, and the need to balance charges for electrical neutrality (Britto and Kronzucker 2002; Balkos et al. 2010). Some NO_3^- may be formed by nitrification of NH_4^+ in the oxygenated rhizosphere and at the floodwater–soil interface. Kronzucker et al. (1998a, b, 1999, 2000) have found that lowland rice can be exceptionally efficient in absorbing NO_3^- compared with other species, raising the possibility that rice growing in flooded soil may absorb significant amounts of NO_3^- formed in the rhizosphere. This is important both because of the potential physiological benefits to the plant, and because NO_3^- formed in the rhizosphere is otherwise lost through denitrification in the soil bulk.

Three lines of evidence from Kronzucker et al. (1999, 2000) suggest unusually efficient NO_3^- absorption. First, steady-state influx of NO_3^- and NH_4^+ followed Michaelis–Menten kinetics over the relevant concentration range, and V_{max} for NO_3^- was some 40 % larger than that for NH_4^+ (8.1 versus 5.7 μmol g^{-1} h^{-1}) and

K_m 50 % smaller (26 ± 6 versus 51 ± 18 μM). Second, induction of the root NO_3^- transporters following its re-supply to plants deprived of NO_3^- for 24 h was exceptionally rapid, peaking within 2 h. For comparison, in barley—which is considered one of the most efficient NO_3^- users—full induction takes up to 24 h (Kronzucker et al. 1997). Third, sub-cellular pool sizes and fluxes, estimated from the kinetics of ^{13}N efflux out of labelled roots, indicated highly efficient NO_3^- use: while similar proportions of incoming NH_4^+ and NO_3^- were channelled into assimilation and to the vacuole, the proportion of NO_3^- translocated to the shoot was larger than for NH_4^+ and that lost through efflux out of the roots was smaller.

The extent of NO_3^- absorption by soil-grown plants will depend on its rate of formation and loss in the rhizosphere. Kirk and Kronzucker (2005) have shown with a model that substantial quantities of NO_3^- can be produced and taken up through nitrification in the rice rhizosphere. Their model considers rates of O_2 transport away from a root and its simultaneous consumption in biotic and abiotic processes; transport of NH_4^+ towards the root and its consumption in nitrification and uptake at the root surface; and transport of NO_3^- formed from NH_4^+ towards the root and its consumption in denitrification and uptake by the root. A sensitivity analysis showed that rates of NO_3^- uptake can be comparable with those of NH_4^+ under realistic field conditions. Also rates of denitrification and subsequent loss of N from the soil remain small even where NO_3^- production and uptake are considerable.

As a result of losses by volatilisation, recoveries of N from mineral fertilisers applied to flooded rice fields are very variable, ranging from 20 to 60 %, with average recoveries between 30 and 40 % in most areas (Peng et al. 2010). Volatilisation occurs by conversion of NH_4^+ to NH_3 in the floodwater linked to high floodwater pHs induced by algae (Sect. 3.1), and by nitrification–denitrification. Recoveries are greatly improved if fertiliser applications are split carefully to match crop demands, as is necessary to realise agronomic yield potentials (Peng et al. 2010).

6.2.2 Phosphorus

In general the changes in soil physico-chemical conditions following flooding result in P becoming more soluble, and so P availability rarely limits rice growth, at least in irrigated systems (Dobermann and Fairhurst 2000; Kirk 2004). In rainfed rice, rapid drying and re-oxidation of the soil can result in the P becoming very insoluble (Brandon and Mikkelsen 1979; Willett 1979; Huguenin-Elie et al. 2003). Re-oxidised Fe(II) compounds may be precipitated in poorly crystalline forms with large specific surface areas, on and in which P becomes immobilised. Hence upland crops grown in rotation with rice frequently suffer P deficiency even though crops on similar soils not used for rice grow healthily. The problem is in part also due to disruption of mycorrhizal networks during flooding (Ellis 1998).

The electrochemical changes and dissolution of soil solid phases in redox reactions following flooding tend to make P more soluble. An interesting question

is how access of P to the root is affected by iron oxide precipitation on and near the root. Amorphous $Fe(OH)_3$ has a large adsorbing surface and might be expected to immobilise P, impeding its access to the root. However, acidification of the rhizosphere might be expected to make P more soluble. Saleque and Kirk (1995) measured concentration profiles of P and other root-induced changes near planar layers of rice roots growing in a highly weathered P-deficient soil. They found that 90 % of the P taken up was drawn from acid-soluble pools, probably associated with Fe(II) carbonates and hydroxides. There were also narrow zones of P accumulation in an alkali-soluble pool which coincided with zones of $Fe(OH)_3$ accumulation near the roots. The zone of P depletion coincided with a zone of acidification. Kirk and Saleque (1995) showed with a model that the acidification and the P-solubilising effect of acidity in the soil were sufficient to account for the P mobilised and absorbed by the roots. This is an extreme example, involving particularly large pH changes. But it indicates the magnitude of the effects that are possible.

Phosphorus deficiency is more prevalent in rainfed rice and aerobic soil conditions. Wissuwa and colleagues (Wissuwa and Ae 2001; Chin et al. 2011; Gamuyao et al. 2012) have explored the genetics and mechanisms of rice tolerance of P-deficient aerobic soils. They identified a major quantitative trait locus (QTL) for tolerance, *Pup1*. *Pup1* is largely absent from irrigated rice varieties but conserved in varieties and breeding lines adapted to drought-prone environments (Chin et al. 2011). It appears to facilitate root growth under P stress by blocking the response to P stress that results in decreased root growth (Gamuyao et al. 2012).

6.2.3 Zinc

The changes in soil chemistry following flooding result in Zn being immobilised in very insoluble forms, even though total Zn contents are generally non-limiting (Kirk 2004; Impa and Johnson-Beebout 2012). This may be linked to high soil pH and excess bicarbonate, such as in the calcareous soils of the Indo-Gangetic plains of India and Bangladesh, but it also occurs in perennially wet, young non-calcareous soils, and in peats and coastal saline soils (Kirk 2004). After N, Zn deficiency is the second most important nutrient disorder in rice, affecting up to 50 % of rice soils globally (Dobermann and Fairhurst 2000). Zinc deficiency in human populations subsisting on rice is also widespread (IRRI 2010).

There is large variation in the rice germplasm in tolerance of Zn-deficient soils (Quijano-Guerta et al. 2002; Wissuwa et al. 2006). At least three mechanisms, operating together or separately, confer tolerance of seedling-stage Zn deficiency: (1) enhanced Zn uptake by secretion of Zn-chelating phytosiderophores (Arnold et al. 2010; Widodo et al. 2010; Ptashnyk et al. 2011); (2) maintenance of new root growth under low Zn and high HCO_3^- concentrations (Widodo et al. 2010; Rose et al. 2011); and (3) prevention of root damage by oxidative stress linked to high HCO_3^- concentrations (Frei et al. 2010; Rose et al. 2011, 2012).

The role of phytosiderophores (PS) in Fe uptake by grasses is well established, but their involvement in Zn uptake is debated (Suzuki et al. 2008). Three lines of

evidence support the PS mechanism in rice. Firstly, Widodo et al. (2010) found enhanced secretion of the PS deoxymugineic acid (DMA) by a Zn-efficient rice line from a QTL mapping population. The tolerant line took up 50 % more Zn in Zn-deficient soil and this coincided with increased expression of putative ligand-efflux genes in the roots. Second, Arnold et al. (2010) found evidence supporting the PS mechanism based on differences in Zn stable isotope fractionation in the same tolerant and intolerant rice lines. They found a preference for heavy ^{66}Zn in the tolerant rice line grown in soil, but not in the intolerant line; among alternative explanations, this could only be explained by a Zn-complexation process and uptake of complexed Zn by the roots. By contrast, rice grown in nutrient culture showed a slight Zn bias, consistent with kinetic fractionation during membrane transport of free Zn^{2+} ions (Weiss et al. 2005). Third, Ptashnyk et al. (2011) developed a mathematical model of PS-mediated Zn uptake, allowing for root growth, inter-root interaction, diurnal variation in PS secretion, decomposition of the PS in the soil, and the transport and interaction of the PS and Zn in the soil. It showed that (a) measured PS secretion rates were sufficient to explain measured Zn uptake rates and differences between rice genotypes and (b) there was an important interaction with rooting density, but (c) diurnally varying PS secretion had little effect on uptake.

6.2.4 Iron Toxicity

Iron toxicity is peculiar to flooded soils. It is a syndrome of disorders associated with the occurrence of Fe^{2+} in the soil solution. It happens over a wide range of Fe^{2+} concentrations, from 1,000 mg L^{-1} to only 10 mg L^{-1} in soils with poor nutrient status—especially of P or K—or with respiration inhibitors such as H_2S (van Mensvoort et al. 1985; Sahrawat 2004; Becker and Asch 2005). The effects include internal damage of tissues due to excessive uptake of Fe^{2+}; impaired nutrient uptake, especially of P, K, Ca, and Mg; and increased diseases associated with imbalanced nutrition. Three main groups of Fe toxic soils are distinguished (van Mensvoort et al. 1985): acid sulphate soils, in which extremely large concentrations of Fe^{2+} in the soil solution arise as a result of the soils' peculiar mineralogy; poorly drained sandy soils in valleys receiving interflow water from adjacent higher land with highly weathered sediments; and more clayey, acid, iron-rich soils in sediments derived from highly weathered soils and which develop iron toxicity without interflow. Where Fe toxicity is associated with interflow the concentrations of dissolved Fe in the upwelling water have been found to be too small to account for the large concentrations of Fe^{2+} in the root zone, and most of the Fe^{2+} is apparently formed in situ. Therefore the interflow aggravates toxicity by some mechanism other than bringing in Fe^{2+}, possibly involving depletion of other nutrients and upsetting the plant's ability to exclude Fe.

In iron toxic soils, oxidation of Fe^{2+} in the rhizosphere is an important means of excluding it from the root. However, in acid soils with small pH buffer powers, the acidification accompanying Fe^{2+} oxidation may result in impaired access of

nutrient cations to roots (Kirk and Bouldin 1991; Razafinjara 1999). This is because the overall concentration of the soil solution in a flooded soil depends largely on the concentration of HCO_3^- buffered by dissolved CO_2. Therefore, if the pH close to the root decreases below about 6.0, the concentration of anions in solution also decreases and so the concentration of cations in solution and hence their rate of diffusion to roots must decrease. Bouldin (1989) gives calculations of this effect. High rates of Fe^{2+} oxidation and associated H^+ generation result in a low pH in the rhizosphere, especially if the soil is already acid or has a small pH buffer power. Hence the need to exclude toxic Fe^{2+} from the root may impair the absorption of nutrient cations by the root. Consistent with this the symptoms of iron toxicity are often alleviated by applications of K salts.

A further complication is that the lowering of the rhizosphere pH and consequent depression of HCO_3^- mean that any Fe^{2+} entering the root will be accompanied by a proportion of Cl^- or SO_4^{2-} rather than HCO_3^-. When Fe^{2+} enters with HCO_3^-, the acidity generated in Fe^{2+} oxidation in the plant is neutralised by conversion of HCO_3^- to CO_2, which is assimilated or lost. However when Fe^{2+} enters with a non-volatile anion, Fe^{2+} oxidation will produce the equivalent amount of free H^+ in the plant, with damaging effects on plant tissues (van Mensvoort et al. 1985).

7 Conclusions

The present and companion (Colmer et al. 2013) papers emphasise genotype-by-environment interactions in *Oryza sativa* resulting in adaptation to a huge range of flooded soil conditions. The remarkable adaptability of *Oryza sativa* to a large range of taxing environments is presumably related to allelic diversity in its small genome, allowing subtle variation in responses to water regimes ranging from free draining to floodwaters many metres deep. This diversity can be employed in mechanistic studies, as well as in plant breeding. The rice gene pool, so eminently adapted to flooded environments, is the key to elucidating the mechanisms of adaptation. It is far better suited to this than the relatively flooding-intolerant species *Arabidopsis thaliana*, which is often the default choice for molecular studies of adaptive mechanisms.

This chapter has considered the most universal feature of the various rice ecosystems: reduced anoxic soils with their large range of inorganic and organic toxins. After 70 years of research we now have a coherent picture of the essential ventilation of the plants roots and its associated rhizosphere, as well as an understanding of acquisition of essential nutrients and prevention of entry of toxic compounds. Whether the 'avoidance' of O_2 deficiency in either tissues or rhizosphere is always effective is less clear. Even in solution culture, O_2 deficiency might occur in root tips and no data are available to evaluate possible O_2 deficiencies of roots in soils of very negative redox potential. To what extent nutrition is dependent on lateral roots needs further elucidation. Similarly, further work is needed on whether the barrier to O_2 loss, along most of the primary root

system, restricts nutrient uptake and how effectively it excludes toxins derived from the anoxic, reduced soil.

The extreme redox gradient across the rhizosphere of rice and other wetland plants means that the soil the root 'sees' has a much higher redox potential (i.e. less negative) than the bulk soil. This has important consequences for the transformations and uptake of nutrients and toxins. Experimental and modelling studies over the past few decades have provided a good understanding of these processes and their consequences. This understanding is beginning to be exploited in rice breeding programmes for tolerance of mineral deficiencies and toxicities. Here again the sequenced rice genome and other genetic resources in rice make it a model plant for future work.

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Physiological Mechanisms of Flooding Tolerance in Rice: Transient Complete Submergence and Prolonged Standing Water

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'Reductionism works in research provided there is a dynamic interchange between different levels of complexity as knowledge develops' (paraphrased from Crick 1994)

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Abstract Partial or complete submergence of shoots of rice (*Oryza sativa* L.) poses a dual challenge: the roots have to function in anoxic soil and gas exchange between shoots and air becomes restricted to a small aerial portion or is abolished during complete submergence. Adaptation of roots to anoxic and chemically reduced waterlogged soils was reviewed by Kirk et al. (Prog Bot, 2014). With deeper floods the O_2 provision to the roots may decline, because there is a high resistance for gas exchange between floodwater and the submerged part of the foliage. Floodwaters differ greatly in light levels and CO_2 concentrations, thus restricting underwater photosynthesis by varying degrees. During the day, underwater photosynthesis largely determines the O_2 concentrations within submerged rice, whereas, at night, tissue O_2 declines, particularly so in roots. Deepwater rice establishes a 'snorkel' via elongation of aerenchymatous internodes and leaf sheaths; these responses are triggered by ethylene, which acts on two *Snorkel* genes encoding ethylene-responsive factor (ERF) transcriptional regulators to elicit the action of gibberellin. In addition, aquatic roots emerge from stem nodes. Perversely, pronounced shoot elongation can be catastrophic for lowland rice completely submerged during transient floods. In these circumstances tolerance is underpinned by suppression of elongation by *SUB1A-1*, an ERF transcriptional

regulator that blocks ethylene responsiveness. However, many aspects of survival during transient complete submergence remain unclear, such as the role of carbohydrate depletion, photosynthesis under water, and anoxia tolerance in roots. After desubmergence, possible injury to shoots from water deficits and free radicals also requires further elucidation. This review is focused on the evaluation of the physiological mechanisms involved in the acclimation–adaptation of rice to these floods.

List of Abbreviations

ADH	Alcohol dehydrogenase
PDC	Pyruvate decarboxylase
QTL	Quantitative trait loci; regions of DNA containing or linked to the genes that underlie a quantitative trait (i.e. a phenotype, such as submergence tolerance)
ROS	Reactive oxygen species
SUB1	A major QTL on chromosome 9 of rice conferring tolerance of transient complete submergence
SUB1A	The gene conferring submergence tolerance at the *SUB1* QTL region. The allele of *SUB1A* that confers submergence tolerance is called *SUB1A-1*. *SUB1A-1* contains a natural point mutation, as compared with the more common allele *SUB1A-2*. Most *Indica* genotypes have a *SUB1A* gene, whereas *Japonica* genotypes do not. *SUB1A* encodes an ethylene-responsive factor (ERF) transcriptional regulator. *SUB1A-1* contains a point mutation resulting in leaf (mainly sheath) elongation being insensitive to ethylene and the lack of a significant underwater elongation response has been termed 'quiescence', which, together with other associated changes, endows submergence tolerance (described in text, with references)

1 Introduction

This review complements the preceding one (Kirk et al. 2014), which dealt primarily with rice (*Oryza sativa* L.) grown in paddy fields with regulated flooding at 0.05–0.2 m, and emphasised adaption to anoxic and chemically reduced soils. That partner review also gave a general introduction on the various aquatic ecosystems of rice. In most rainfed lowland rice ecosystems floods are deeper than the 0.2 m water depth in irrigated systems; for example floodwaters often exceed 0.5 m, and in some locations (deepwater areas) the depths can be up to 4 m. Further, the flood

duration in these rainfed regions varies from transient short-term (a few days up to 3 weeks) complete submergence to several months of partial submergence such as in deepwater areas (Catling 1992).

This review critically evaluates the physiological mechanisms involved in the adaptation of rice to these floods. The last reviews that focused on the physiology and ecology of submergence tolerance were by Jackson and Ram (2003) and Voesenek et al. (2006). The impressive gains in understanding of the genes involved in the tolerance of rice to flooding have been extensively considered elsewhere (Toojinda et al. 2003; Bailey-Serres and Voesenek 2008; Bailey-Serres et al. 2010; Voesenek et al. 2006; Voesenek and Bailey-Serres 2009). In this review, we only briefly summarise current understanding of the genetics of submergence tolerance to give suitable background to our main physiological theme. The physiological understanding is not only of interest for its own sake, but may also be of value to interpretation of results obtained by microarrays and association mapping. The variations in depth and duration of the floods are further complicated by other characteristics of the floodwaters, to name the most important ones, levels of CO_2, irradiance, and turbulence, which vary widely between locations. So, to further advance our understanding of acclimation phenomena and the key genes that code for adaptive responses to flooding, it is likely that interactions between disciplines of physiology and genetics will assume greater importance.

The two main categories of flooding are (1) long-term partial submergence, when shoot elongation is a prerequisite to success and (2) transient complete submergence for up to 3 weeks, when elongation can be fatal. Further comments on these types of flooding are discussed in Sect. 2. In nearly all rice varieties, flooding triggers some degree of elongation of internodes or leaf sheaths. Adaptation to long-term deep flooding is primarily achieved by stem (i.e. internode) elongation, as mediated by two *SNORKEL* genes. *SNORKEL1* (*SK1*) and *SNORKEL2* (*SK2*) both are ethylene-responsive factor (ERF) transcriptional regulators that control internode elongation (Hattori et al. 2009). This elongation maintains, or re-establishes, emergent (i.e. in air above the water) foliage and so enables photosynthesis in air and facilitates internal aeration (Catling 1992). Mechanisms driving this elongation phenomenon in internodes are discussed in Sect. 4, including the pivotal role of the phytohormone ethylene. Shoot elongation (Sect. 4) and limitations on photosynthesis by the submerged part of the shoot (Sect. 3) have, however, consequences for carbohydrate allocation (Sect. 5.2). In addition, a poorly researched area is the function of the prolific aquatic roots in deepwater rice (discussed in Sects. 3.4 and 5.3).

In sharp contrast to rice in areas with deep prolonged floods for which internode elongation is essential, during transient complete submergence leaf (i.e. mainly sheath) elongation can be catastrophic (Sect. 6). This dramatic contrast in performance depending on duration of the floods is exemplified by the phenotype of rice varieties in which underwater elongation has been curtailed by introgression of the *SUB1* QTL which suppresses ethylene responsiveness. Sub1 varieties are top

performers during transient flooding causing complete submergence, but, like other short-statured genotypes, the first generation of Sub1 varieties performed far worse than deepwater varieties during prolonged flooding, even with water only 50 cm deep (Singh et al. 2011). Performance of rice does not correlate simply with the duration of flooding but also depends on floodwater characteristics such as irradiance, temperature, and CO_2 and O_2 levels, which in turn vary strongly over space and time (Sect. 2) influencing underwater gas exchange (Sect. 3). This great diversity highlights how flooded fields are a 'different world' from the terrestrial environment. We emphasise a few reasonably well-researched cases, but it is probable that more variations in the response of rice, associated with specific environments, will be revealed in due time.

2 Characteristics of Floodwaters

Water chemistry in flash-flood and deepwater areas is highly variable. Carbon dioxide and O_2 concentrations become a complex function of photosynthetic rates of rice and co-habiting organisms, diffusion of these gases between atmosphere and floodwater, turbulence, and uptake or loss of these gases by the submerged foliage. This situation in the overlying water containing O_2 contrasts with that in the bulk soil which soon after flooding becomes anoxic and redox potential declines (Kirk et al. 2014).

There are huge variations in both O_2 and CO_2 concentrations in floodwaters, in distributions with depth, and in the amplitude of diurnal changes (Setter et al. 1987a; Ram et al. 2002; Jackson and Ram 2003; van Eck et al. 2005; Das et al. 2005). Generally, photosynthesis during the day and respiration at night cause diurnal cycles, with O_2 levels high during the day and low during the night and converse patterns for CO_2 (e.g. Fig. 1). In addition, O_2 is highest in the surface water layers and often close to zero near the soil–water interface, while CO_2 shows the opposite pattern.

The large variations in dissolved gases in floodwaters, as discussed in the subsections immediately below, have been used to discuss the disparities observed in the performance of various rice varieties, when evaluated in different geographical regions and seasons (Ram et al. 1999; Setter et al. 1995). In view of the huge field variations in CO_2 and O_2 concentrations in floodwaters, measurements at locations of special interest need to be established on a case-by-case basis.

2.1 Duration of Floods

In this review, in addition to consideration of transient floods resulting in complete submergence of up to 3 weeks, we also use the following conventions to describe

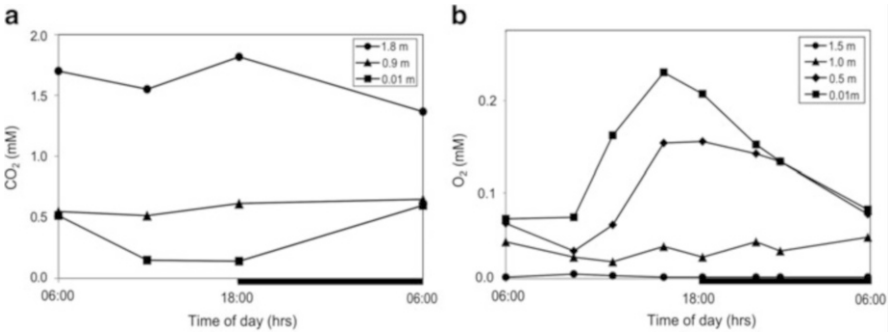

Fig. 1 Diurnal fluctuation of CO_2 and O_2 in a deepwater rice field (1.5–1.8 m) at Maharat, Thailand: (**a**) CO_2; (**b**) O_2. Symbols show the water depths at which the samples were taken. The figure is representative of field surveys taken by Setter and colleagues in Thailand between 1983 and 1986. From Setter et al. (1987a), *Plant*, *Cell & Environment*, with permission from John Wiley and Sons

the range of habitats and rice types in areas with flooding for several months, in that case for most of the time with only partial submergence. All these longer-term floods last more than 1 month and often several months. The three categories that we distinguish are moderate deep water (0.2–0.5 m; also referred to as 'stagnant flooding', medium-deep or semi-deep in some publications, including those by IRRI staff), deep water (0.5–1 m), and very deep water (>1 m, reaching depths of several metres) (modified from Catling 1992). Deepwater rices have been subdivided into 'tall' and 'floating' types. 'Tall rice' is intrinsically tall and grown in areas with 0.5–1 m water and their elongation reaches a much shorter length than rice in the very deep water areas. Most rice genotypes growing in very deep water have often been called 'floating rice' as the internodes elongate strongly to produce stems up to several metres in length (Catling 1992), but the term 'floating rice' tends to be confusing since, as Catling (1992) pointed out, most rices of this type remain anchored in the sediment—'floating' refers then to the buoyant elongated stems being supported by the water. During partial submergence by very deep floods, and particularly during complete submergence, severe restraints of gas exchange by the foliage are added to the critical challenge of roots coping with anoxic and chemically reduced soils (Kirk et al. 2014).

2.2 *Irradiance and Turbulence Influence Exchange of Gases Between Submerged Foliage and Floodwaters*

Irradiance will be discussed first since it affects photosynthesis, which by removing CO_2 and evolving O_2, is the main driver of the diurnal cycles and spatial distributions of these gases dissolved in the floodwaters.

Irradiance in the water varies greatly and is described by the 'attenuation constant' and depth, $I_z = I_0\ e^{-kz}$. I is irradiance, I_0 is incident irradiance, z is depth, and k is the attenuation constant (Jackson and Ram 2003). k is about 0.3 in clear water and can be as high as 0.9 in turbid water (Jackson and Ram 2003). During flash floods in India, incident radiation was reduced to 50 % at water depths which varied between 0.07 and 0.39 m at different locations (Ram et al. 1999). Similarly, in a study of deepwater rice in Thailand, irradiance which would result in 50 % of maximal photosynthesis was attained at different locations between 0.15 and 0.7 m depths (Setter et al. 1987a). In general, attenuation may be related to the canopy, but also to turbidity associated with phytoplankton, algae, and silt. Silt densities of 0.2–0.4 % (w/v) were recorded following flash flooding in some locations (Das et al. 2009).

Turbulence is a crucial factor determining gas exchange between floodwaters and plant tissues, by greatly reducing the thickness of the unstirred layer (i.e. diffusive boundary layer) adjacent to plant surfaces. Detail of the crucial importance of turbulence is given in Sect. 3. Pronounced turbulence will also mix the floodwater from different depths and so will dampen the diurnal cycles in O_2 and CO_2, while providing a larger CO_2 source in the surface layers of water with the highest irradiance, as well as providing O_2 to the deeper water layers. Therefore, any conditions (e.g. flowing water) which enhance turbulence within the floodwaters are likely to improve performance of submerged, or partially submerged, rice.

2.3 Variations in Concentrations of Dissolved Gases in Floodwaters

2.3.1 Carbon Dioxide

Carbon dioxide concentrations in floodwaters are of key importance to the carbon balance of submerged shoots. With very high irradiance and turbulence and 1 mM CO_2, relative growth rates of shoots of submerged rice, over 4–6 days, reached 80 % of the rate measured in air (Setter et al. 1989). However, high irradiance and turbulence seldom occur in the field, although these limitations can be compensated for if CO_2 in the floodwater would exceed 1 mM. These higher CO_2 concentrations (e.g. up to 3.2 mM) occur in some floodwaters (Jackson and Ram 2003). Further, in two out of three locations with deepwater rice in Thailand, CO_2 concentrations at midday at 0.1, 1.0, and 1.8 m below the water surface were 0.1–0.3, 0.6, and 1.0–1.8 mM, respectively (Setter et al. 1987a). Since irradiance at 1.8 m depth is usually low, photosynthesis would be light-limited and the CO_2 at these lower depths would be mainly a source for leaves in the upper water layers.

The diurnal cycle in CO_2 concentrations (Fig. 1a) gives rise to a diurnal cycle in pH of floodwater, which is important since the rise in pH during the day will amplify the decrease in CO_2 concentration caused by its consumption in photosynthesis. The pH may increase from near neutral at night to 10 during the day (Mikkelsen and de Datta 1978). This rise in pH is most easily explained by the notion of the strong ion difference (Stewart 1983; Gerendas and Schurr 1999): CO_2 removal, and hence decreases in HCO_3^-, during the day will increase the 'strong ion difference', the difference between equivalents of cations and anions (excluding H^+ or OH^-) and therefore increase the pH. The decrease in dissolved CO_2 is then further amplified because the increase in pH will shift the equilibrium of CO_2/HCO_3^- (the main buffer in the floodwater) towards HCO_3^-. This would further limit photosynthesis in submerged rice, which uses CO_2, not HCO_3^-, as the carbon source (Sect. 3).

2.3.2 Oxygen

Oxygen concentrations in the floodwater in rice fields vary widely between day and night, with depth of the water and with locations. During the day, the risk of O_2 deficiency in the lower shoots and roots is less because O_2 evolved during photosynthesis in the upper leaves would diffuse through the aeration network and along gas films on leaves. High O_2 concentrations in the water by day would diminish O_2 diffusion from the shoots into the floodwater and hence improve the gradient for O_2 flow to the lower parts of the shoots and the roots, whereas at night photosynthesis ceases and the O_2 concentration in the floodwater also declines as it is consumed in respiration. Thus, during the night O_2 deficiency might occur, especially in roots (Waters et al. 1989; Winkel et al. 2013).

To gauge the importance of the floodwater O_2 concentrations to the shoots, we note that in rice coleoptiles in turbulent solutions, respiration rates at 0.03–0.05 mM O_2 were half of the maximum rate (Atwell and Greenway 1987). In the field, starting in late afternoon when the highest O_2 levels are usually reached, O_2 was 0.14–0.27 mM at 0.1 m from the air–water interface and 0.03–0.2 mM at 0.9 m depth, indicating sufficient O_2 to maintain maximal respiration rates, at least in thin tissues. Lower O_2 concentrations are often found near the floodwater–soil interface; at water depths between 1.4 and 1.8 m, O_2 was near zero and remained so during the entire diurnal cycle in three out of seven profiles in these deep floodwaters (Setter et al. 1987a; Ram et al. 1999). Quite a different picture is found in other locations. Even at dawn, turbulent floodwater of submerged rice contained approximately 0.17 mM O_2 at the soil–water interface (Ram et al. 1999, water depth 0.8 m) and tracked at the same levels as at 0.1 m depth during the diurnal cycle. A similar profile, with O_2 at 0.13–0.19 mM, was found in one location on an acid sulphate soil in a deepwater rice field in Thailand (Setter et al. 1987a, 1.4 m water depth).

Algal blooms can substantially affect the concentrations of dissolved O_2 and CO_2 in floodwaters. As one example, O_2 in the floodwater rose at midday to as high as 0.5 mM in a flooded field with a heavy algal bloom (Ramakrishnayya et al. 1999), while the bloom would of course also lower the CO_2 concentration. Such conditions favour photorespiration, further discussed in Sect. 3.5.

2.3.3 Ethylene

Data on ethylene in floodwaters are scarce. Estimates ranged between 0.07 and 2.41 Pa in deepwater rice fields in Thailand (Setter et al. 1988a). The only data we know on ethylene within submerged rice are from 11-day-old seedlings submerged in nutrient solution where the internal ethylene was 0.7 Pa (Jackson et al. 1987). Ethylene is central to initiation of elongation, whether in internodes of deepwater rice or in leaves of lowland rice during transient complete submergence (Sects. 4.2 and 6). However, at low levels of ethylene, there might be antagonism from high CO_2. This interaction was shown for rice leaves in glasshouse experiments where addition of 1 kPa CO_2 to a gas phase around the leaves containing 0.35 Pa ethylene substantially reduced the chlorosis and decreased leaf elongation relative to ethylene-treated plants without this high CO_2 (Jackson et al. 1987). Further, if interactions did occur between ethylene and CO_2 in rice in flooded fields, the antagonism would be more pronounced at night, in view of the higher CO_2 during dark periods. Another question is to what extent ethylene in the shoots would be elevated by complete submergence, i.e. by preventing direct ventilation to the atmosphere, and whether any elevated concentrations would intensify chlorosis. Neither the ethylene concentration in field-grown shoots nor the dose–response of internal ethylene and chlorosis is known. There have been no further studies on this aspect, either in the field or in the laboratory.

3 Gas Exchange Under Water

Gas exchange under water is relevant to both partial and complete submergence and is therefore discussed first.

When shoots are submerged there is a huge increase in effective resistance to CO_2 and O_2 exchange, compared with the situation in air, resulting from the slow diffusion across aqueous diffusive boundary layers (i.e. unstirred layers). Diffusion of gases (e.g. CO_2 and O_2) dissolved in water is 10,000 times slower than gas diffusion in air (Armstrong 1979). Key factors determining O_2 and CO_2 movements are (1) the concentration gradients between the tissues and surrounding water, and (2) the amount of turbulence in the bulk floodwater and thus the thickness of the boundary layers as a major component of the resistance to underwater gas exchange (Colmer et al. 2011). Within plants the concentrations of these gases are also dependent on net photosynthesis as affected by irradiance, and in the dark by

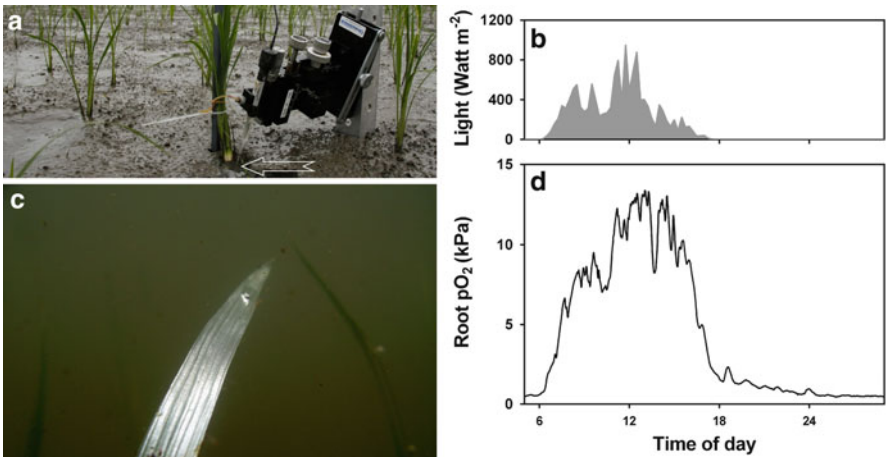

Fig. 2 (**a**) Microelectrode set up in an experimental field pond at the International Rice Research Institute (IRRI, The Philippines) used by Winkel et al. (2013) to measure O_2 in roots during submergence. The fine glass electrode has been inserted into an adventitious root (1 cm below the root–shoot junction) and re-covered with soil, and then the plants were submerged. (**b**) Incident light during the measurements. (**c**) Silver shiny appearance of a leaf of submerged rice in the field pond, caused by the reflection of light owing to the gas film. (**d**) Recording with time of O_2 in the root cortex showing O_2 present during the day, but decreasing to very low values during the night. Data extracted from Winkel et al. (2013), *New Phytologist*, with permission from John Wiley and Sons. Photographs taken and figure prepared for us, by Ole Pedersen

respiration. In rice, an important feature that reduces the resistance to underwater gas exchange is gas films on leaf surfaces (Raskin and Kende 1983; Pedersen et al. 2009), as discussed in the next section (Sect. 3.1). Rice is reliant on CO_2 as its inorganic carbon source, and it cannot utilise HCO_3^- (Setter et al. 1989). By contrast, many aquatic species can also utilise HCO_3^- (Maberly and Madsen 2002) which is a substantial advantage in aquatic habitats, since when only CO_2 is absorbed, the pH of the floodwaters tends to increase during the day, hence shifting the HCO_3^-/CO_2 equilibrium further towards HCO_3^- (Sect. 2.3.1).

3.1 Gas Films on Leaves

Gas films occur on superhydrophobic leaf surfaces (Fig. 2c) resulting from microstructures and waxes which repel water sufficiently to form/retain a thin gas layer when under water (reviewed by Colmer et al. 2011). Superhydrophobic leaf surfaces are common amongst terrestrial wetland plants and are retained during submergence (Colmer and Pedersen 2008), including on leaves of rice (Raskin and Kende 1983; Setter et al. 1989; Pedersen et al. 2009). It should be noted that leaf

surface hydrophobicity also serves various functional roles, even when there is no flooding, in many terrestrial plants (Neinhuis and Barthlott 1997; Brewer and Smith 1997).

The presence of gas films on the surfaces of submerged leaves (Fig. 2c) reduces the resistance to gas exchange compared to when such films are absent. For rice, resistance to CO_2 uptake by leaf segments was approximately five times less with gas films intact than when the films had been artificially removed (Pedersen et al. 2009). Leaf gas films also facilitate O_2 exchange with the floodwater (Pedersen et al. 2009), a function analogous with body surface gas layers (termed 'plastron') on some aquatic insects (Raven 2008; Pedersen and Colmer 2012). For leaves with a waxy cuticle the stomata need to remain open for gas films to be effective in promoting gas exchange (Raskin and Kende 1983; Pedersen et al. 2009) and supporting evidence for this situation is the high stomatal conductance measured immediately after desubmergence of rice (Smith et al. 1988).

The usual interpretation of the benefit of the gas films has been that these provide a large gas-to-water interface (Raskin and Kende 1983; Setter et al. 1989; Pedersen et al. 2009; Pedersen and Colmer 2012). However, we now propose an alternative explanation based on modelling of resistances in the pathway for inward O_2 diffusion (Armstrong Turner and Beckett, unpublished). The slower diffusion into the leaves without gas films is attributed to a high resistance because the vast majority of O_2 fluxes through the water boundary layer follow oblique paths from the bulk water to the stomata, in contrast to leaves with gas films where the O_2 moves perpendicular through the aqueous boundary layer into the gas film and then along the leaf surface, through the negligible resistance in the gas phase to stomata. This unpublished modelling indicated that gas films would lower the total resistance between the bulk water and the stomata by six- to sevenfold (Armstrong and colleagues, unpublished), i.e. reasonably close to the experimentally determined differences in fluxes between leaves with gas films intact or artificially removed (e.g. Colmer and Pedersen 2008; Pedersen et al. 2009; Winkel et al. 2013).

The advantage of gas films on leaves was demonstrated in experiments comparing leaves with and without gas films; the latter were brushed with dilute Triton X-100 to disrupt the hydrophobic layer and hence prevent gas film formation (Raskin and Kende 1983; Pedersen et al. 2009; Winkel et al. 2013). Evidence includes an experiment with rice submerged for 7 days, with irradiance (PAR) 500–600 μmol m^{-2} s^{-1} and CO_2 concentration of 0.2 mM. Plants with gas films grew as well as plants with shoots in air, but during submergence removal of the gas film reduced relative growth rate of the shoots (dry weight basis) by 60 % with no dry weight increments in the roots at all, while sugar concentrations were reduced in the leaves by 20 % and in roots by 50 % (Pedersen et al. 2009). Consistently, removal of the gas films increased apparent resistance to CO_2 uptake by fivefold and reduced net photosynthesis (Pedersen et al. 2009). Underwater photosynthesis, measured by $^{14}CO_2$ fixation over 15 min after submergence of excised rice leaves, was reduced by 13-fold when gas films were removed (Raskin and Kende 1983). Further, and probably as a result of less photosynthesis, at the end of complete submergence for 90 h, plants with gas films removed had much less chlorophyll and

protein than plants with gas films remaining; reductions for leaves with and without gas films were, respectively, 25 and 50 % for chlorophyll and 10 and 65 % for protein (Raskin and Kende 1983, both on a fresh weight basis). The alternative explanation for the more severe chlorosis in leaves without gas films may be high ethylene due to less ventilation (Sect. 2.3.3). Finally, in addition to increased CO_2 uptake for photosynthesis during light periods, gas films also enhance O_2 exchange between the floodwaters and the submerged canopy, with O_2 enrichment of the floodwaters during the day and O_2 movement from floodwaters into submerged plants during the night, as demonstrated by an experiment in darkness in which the gas films of rice leaves were removed, which caused internal O_2, measured in the roots, to decline from about 3.4 to 0.1 kPa (Pedersen et al. 2009).

Gas films are common to many wetland plants with emergent leaves, termed 'terrestrial wetland plants', which include rice (Colmer et al. 2011). This group typically has, most of the time, many emergent leaves and was contrasted with 'submerged aquatic plants' (Colmer et al. 2011). These submerged aquatic plants possess quite different leaf traits for enhancement of gas exchange under water (Maberly and Madsen 2002; Colmer et al. 2011); they have thin or absent cuticles, and therefore can absorb CO_2 over the full leaf surface, as well as possessing other traits such as carbon concentrating mechanisms and use of HCO_3^-. Some typically emergent wetland species display leaf plasticity in response to submergence, such as having much reduced cuticles, which enhances CO_2 uptake for photosynthesis when under water (Mommer and Visser 2005). Thin cuticles, however, become a major disadvantage when floodwaters recede; in *Rumex palustris* all leaves formed under water, except the youngest, wilted and withered within a few hours after desubmergence (Chen et al. 2011).

A second function of gas films occurs when leaves are emergent, as then there will be long-distance gas transport between the air and the lower parts of the foliage, within the surface films. The surface gas layers add considerable capacity for gas movement in addition to that via internal lacunae in the leaf, so that O_2 movement from the air to the submerged portions of the shoots, such as stem nodes to which the leaves are attached, is enhanced (Raskin and Kende 1983). These authors proposed that the O_2 flow within the gas films was mainly along pressure gradients; this conclusion was based on evidence from short-term experiments (Raskin and Kende 1983). However, using modelling, Beckett et al. (1988) demonstrated that in rice there is no long-term benefit from any convective flow. The key reason for this outcome is that there is no through flow, so any bulk (i.e. convective) flow will diminish the concentration gradients and therefore merely replace the diffusive flux, not augment the total flow (Beckett et al. 1988). This conclusion on rice contrasts with some wetland species, which have through flow; this allows convective flow to be far superior to diffusion along concentration gradients. The important conclusion on rice was based on rigorous mathematical modelling and experimental analyses (Beckett et al. 1988).

Next we discuss the importance of the gas films to O_2 uptake from floodwaters at night (Sect. 3.2), being a relatively simple case, and then the events during the day when CO_2 fixation in photosynthesis *ipso facto* also affects the O_2 regime (Sect. 3.3), not only of the plant but also of the floodwaters.

3.2 Importance of Gas Films to O_2 Supply During Darkness

During the night, the importance of gas films on leaves to the supply of O_2 to completely submerged rice was shown in the field for roots in an anoxic soil (Winkel et al. 2013). Oxygen in the cortex of adventitious roots at 1 cm below the root–shoot junction was measured using O_2 microelectrodes (Fig. 2a; Winkel et al. 2013). Obtaining such data in the field was not trivial and required specialised equipment. A positive correlation ($r^2 = 0.73$) between the O_2 concentration in the floodwater and that in the cortex of roots in the soil indicated that at night the floodwater was an important O_2 source to submerged rice. Further, when the gas films had been removed, O_2 in the cortex of the basal part of the roots dropped below detection, compared to between 0.24 and 0.42 kPa O_2 for plants with leaf gas films (Winkel et al. 2013). This function of the gas films to provide O_2 to roots of submerged rice during darkness was also demonstrated in controlled-environment experiments by Pedersen et al. (2009).

The O_2 levels of 0.24–0.42 kPa measured in the top cm of the roots indicate that even with gas films on the leaves, the deeper parts of the root system might become anoxic during the night (Winkel et al. 2013), as shown in controlled experiments on submerged rice seedlings (Waters et al. 1989). The low O_2 levels so close to the root–shoot junction of submerged rice in the field experiment are probably associated with a large sink for O_2 in the anoxic soil. The overall picture from the field measurements was consistent with earlier experiments with rice in a temperature-controlled glasshouse. When the shoots were submerged, the roots leaked ethanol to the nutrient solution during the night, at a rate of at least 1.4 and 2.3 μmol g^{-1} fresh weight h^{-1}, for floodwater around the shoots at 21 and 10 kPa O_2, respectively (Waters et al. 1989). In the same set of experiments, but in a growth room, roots were in deoxygenated agar; in that experiment root extension ceased when the lights were switched off and commenced again when the lights were switched on again. Radial O_2 loss just behind the root tips, measured using root-sleeving electrodes, demonstrated that photosynthetically produced O_2 moved from shoots to root tips and relieved the night-time anoxia in submerged rice (Waters et al. 1989). These data support the notion that tolerance to O_2 deficiency might be important for submerged rice at least in the roots during nights particularly in the tropics where soil temperatures remain high. However, further investigation, particularly of the root tissues deeper in the soil, is required for field-grown rice, during both long-term partial submergence and transient complete submergence.

3.3 Photosynthesis and Related Changes to Internal O_2 Status

The substantial benefit of the gas films in facilitating gas exchange when under water was also demonstrated by measuring net photosynthesis of rice leaf segments in water with a range of CO_2 concentrations (Pedersen et al. 2009). O_2 evolution

reached a maximum at 0.5 and 2 mM CO_2 in the medium for leaf segments with and without gas films, respectively (Pedersen et al. 2009). These rates of net photosynthesis in water with CO_2 levels enriched above air equilibrium are of relevance since the mean dissolved CO_2 for flash floods is about 1 mM and for deep water and stagnant flooding 0.37 mM (collated by Colmer et al. 2011), but CO_2 in floodwater declines during the day as it is consumed (Sect. 2). The maximum rate of net photosynthesis for submerged rice leaves with gas films was 4 μmol m^{-2} s^{-1} (reached at 0.5 mM CO_2), still well below the rate of 11.3 μmol m^{-2} s^{-1} for leaves in air at ambient CO_2 (0.038 %) and the CO_2-saturated rate in air of 17.8 μmol m^{-2} s^{-1} (Pedersen et al. 2009). Similarly, underwater net photosynthesis of excised leaf segments in laboratory measurements with dissolved CO_2 at levels similar to those measured in a submergence field pond was only 4.5 % of that achieved by leaves in air at ambient CO_2 (Winkel et al. 2013). Yet, the maximum net photosynthetic rate in the submerged rice leaves was still eightfold higher than the rate of respiration (Pedersen et al. 2009); i.e. in this particular experiment there would have been carbohydrate produced, accounting for the growth of plants in an associated submergence experiment in a controlled-environment room. Consistently, rice submerged in water flushed with 17 kPa CO_2 (a very high CO_2 supply) for 4–6 days attained the same shoot relative growth rate as plants with shoots in air (Setter et al. 1989). Rates of photosynthesis by intact leaves of rice submerged in the field would depend upon light, turbulence, and CO_2 concentration in the floodwater.

Another imponderable is whether rice can sustain substantial photosynthesis based on CO_2 derived via the aerenchyma from the sediment. Flooded soils and sediments are often rich in CO_2 (Greenway et al. 2006; Winkel and Borum 2009). Comprehensive studies on five aquatic species showed that the contributions to photosynthesis, based on CO_2 derived from the sediment, varied from very substantial to zero (Winkel and Borum 2009). These authors list four factors which favour use of the sediment source of CO_2: large aerenchyma, short stature, a shoot-to-root ratio under 1.0, and absence of impermeable layers to gases in the roots. There are no data on use of sediment CO_2 for rice, but a substantial contribution seems unlikely, since only the requirement of large aerenchyma is sure to apply. Other factors which might be favourable for young plants is their short stature and low shoot-to-root ratios of 1.1 for seedlings and 2.0 for tillering plants (Pearson and Jacobs 1984). Even at a shoot-to-root ratio of 2.0 in aquatic species there was some contribution to photosynthesis by CO_2 derived from the sediments (Winkel and Borum 2009). Although the main root axis contains a barrier to gas diffusion, the lateral roots are permeable (Kirk et al. 2014) possible contributions to photosynthesis by CO_2 derived from the sediments requires assessment in future research.

During submergence, photosynthesis is also of benefit to the O_2 supply of the plants. In controlled-environment experiments on submerged rice, the O_2 concentration at 20–25 mm behind the tip of adventitious roots of 80–120 mm in length in deoxygenated agar medium was 3.4 kPa during the dark and 13.5 kPa when the shoots were provided with light (means in Table 1 of Pedersen et al. 2009). Similarly, field measurements at 1 cm below the shoot–root junction of submerged

rice showed that O_2 was 0.24 kPa at night but rose to 14 kPa during the day (Winkel et al. 2013). Even so, 15 % of the variation in O_2 levels within the roots could still be attributed to the dissolved O_2 in the floodwater. The rise in O_2 in the floodwater during the day would reduce the O_2 movement from the shoots to the floodwater, and so result in a larger O_2 gradient between shoot and root (Winkel et al. 2013). Interestingly, cloud cover decreased root O_2 by 50 % and this response was consistent with an approximately 40 % decrease in O_2 at the epidermis of root tissue just behind the root tip of submerged rice in a growth cabinet experiment when irradiance was reduced from 900 to 450 μmol m^{-2} s^{-1} (Waters et al. 1989). Routine evaluation of photosynthesis and resulting tissue O_2 status in field conditions would be far too time-consuming, at least with current methods. However, when the much-easier-to-measure CO_2 concentrations and irradiance levels of the floodwaters are known, CO_2- and light-response curves (e.g. Pedersen et al. 2009; Winkel et al. 2013) could be used to assess potential photosynthetic rates in submerged rice in the field. These assessments would be underestimates if CO_2 derived from sediments enters roots and moves to the shoots providing CO_2 in addition to that from the bulk water. In conclusion, even low rates of photosynthesis, by providing some photosynthate and O_2, might postpone or prevent the catastrophe, often occurring to plants during these floods (Sect. 6).

3.4 Photosynthesis in Aquatic Roots

Aquatic roots which form at stem nodes of rice in deep water (Suge 1985) might, like in a few other wetland species (Rich et al. 2008, 2012), contain chlorophyll and thus some capacity to photosynthesise. The capacity for light-exposed roots of rice to develop chloroplasts was demonstrated in Armstrong and Armstrong (1994) and an example is shown in Fig. 3; this study assessed adventitious roots that would normally grow in sediments. The chloroplasts in aquatic roots might be of significance as providers of O_2, since the energy requirements of these roots might be considerable in view of their ion absorption and associated metabolism [cf. ion uptake by hypoxic roots discussed in Colmer and Greenway (2011)]. The assessed CO_2-saturated net photosynthesis in aquatic roots of the wetland plant *Meionectes brownii*, measured as net O_2 production, was slightly less than their measured dark respiration (Rich et al. 2011); i.e. in that case a contribution to the carbohydrate balance of the rest of the plant is unlikely. Maximum rates provide a reasonable yardstick for performance at suboptimal levels of CO_2, since half-maximal rates for leaves, stems, and roots were all reached at about the same exogenous CO_2 level (Rich et al. 2011). Despite only having a low photosynthetic capacity, O_2 partial pressures in aquatic roots surrounded by stagnant deoxygenated agar were at 10 kPa in the dark and rose to about 18 kPa when the lights were switched on (Rich et al. 2011). When these roots were severed from the plant, O_2 in the roots in the light remained steady, while in the dark O_2 dropped to zero in less than 2 h (Rich et al. 2011).

Fig. 3 Cross section of an adventitious root of rice showing chloroplast development (*small red spheres* within the cortical cells) after 8–10 weeks of exposure to light (PAR of 40 μmol m^{-2} s^{-1} in nutrient solution culture in a controlled environment. The section was illuminated with *blue light*, so that chlorophyll was fluorescing *red*. The section was taken using a handheld razor blade at 1–2 cm below the root–shoot junction. From Armstrong and Armstrong (1994), *New Phytologist*, with permission from John Wiley and Sons. Photograph provided by Jean Armstrong

The nutrient uptake of aquatic roots may be critical to the nutrition of deepwater rice. In submerged *Meionectes brownii* the aquatic roots had replaced the sediment root system (Rich et al. 2011, 2012), and visual observation indicates moribund root systems in deepwater rice after long periods of partial submergence (H. Greenway pers. obs.). A major question is to what extent sediment root systems remain functional in deepwater rice?

3.5 Relationship of Underwater Photosynthesis and Submergence Tolerance: Can Underwater Photosynthesis Be Improved?

Underwater gas exchange may be important during both transient complete submergence and long-term partial submergence, yet little is known about the possible contribution of light reaching submerged tissues for tolerance to these floods. The few existing studies, which are on species other than rice, suggest a strong effect by light, since even low levels of irradiance can result in two- to fourfold better survival (Mommer et al. 2004; Vashisht et al. 2011). Importantly, photosynthesis will not only improve aeration during the day, but together with photosynthesis by phytoplankton and algae, enrich the floodwaters with O_2, which will only gradually

decrease during the night, thus also providing for respiration during the dark period (Winkel et al. 2013; Sect. 3.2).

The question arises whether the photosynthetic performance of underwater parts of rice can be further improved. Whether rice germplasm differs for gas film formation and persistence when submerged in field conditions should be evaluated (suggested by Colmer et al. 2011). Gas films can persist over extended periods for submerged rice; at least in an experiment by Setter et al. (1989) at high CO_2 and irradiance, the films persisted for 84 days (H. Greenway, pers. obs.). In a recent field experiment on rice in submergence ponds at IRRI the gas films lasted up to 6 days (A. Winkel, unpublished data).

Other approaches might also be considered for potential to improve underwater photosynthesis in rice. Firstly, C4 photosynthesis occurs in some aquatic plants (Maberly and Madsen 2002). So, current efforts to introduce C4 photosynthesis into rice aimed at increasing yields (Sage and Sage 2009; Miyao et al. 2011) might have the side benefit of improving performance of submerged rice; C4 photosynthesis acts as a CO_2 'concentrating mechanism' (Maberly and Madsen 2002), so that CO_2/O_2 in the chloroplasts would become higher, mitigating photorespiration, always a threat when the surrounding water hampers CO_2 entry and O_2 exit from the leaves. The present evidence for possible occurrence of photorespiration in rice during flooding is indirect; O_2 evolution measured in cuvettes declined when the O_2 concentration of the solution reached about 22 kPa, but immediately resumed at the initial high rates when exogenous O_2 was lowered by flushing transiently with N_2 gas (Setter et al. 1989). Further, using several assumptions, the O_2 concentration in the chloroplasts of illuminated rice leaves in floodwater containing 0.2 mM O_2 has been estimated at 0.5 mM (Setter et al. 1989), indicating photorespiration might often be substantial. Gas films on leaves might also mitigate photorespiration via enhanced CO_2 entry and O_2 exit underwater (suggested by Colmer and Pedersen 2008). However, photorespiration and internal O_2 and CO_2 concentrations in submerged rice leaves and the floodwater in such field situations need to be evaluated. A second possibility, being an alternative to C4, would be to complement the C3 photosynthesis in rice with characteristics of CAM-type photosynthesis, which occurs in some specialised submerged plants (Maberly and Madsen 2002; Colmer et al. 2011). CAM plants incorporate CO_2 into malate at night, which is of particular advantage since CO_2 concentrations in floodwaters are approximately three- to fivefold higher at night than during the day (Ram et al. 1999); subsequently the malate is decarboxylated during the day and then fixed during C3 photosynthesis. The beneficial effects of CAM for underwater photosynthesis, including diminished photorespiration, were demonstrated for *Isoetes australis* (Pedersen et al. 2011). Both of these possibilities (C4 and CAM) are speculative and technically challenging and so not assured of success, but are more promising than alternative strategies such as possible attempts to simulate the thin cuticle of leaves of aquatic plants to enhance CO_2/HCO_3^- absorption, since thin cuticles would likely enhance desiccation upon desubmergence.

3.6 Summary

Water qualities which enhance underwater photosynthesis can be of substantial benefit to partially or completely submerged rice. All the same, it is highly unlikely that this gas exchange would be sufficient to sustain high yields. Further acclimation requires regulation of elongation, but in two dramatically different ways, dependent on the flooding regime. In long-term floods lasting several months, elongation is indispensable to achieve or maintain a substantial emergent canopy to photosynthesise in air. Diametrically opposite to these long-term floods, during shorter complete submergence of up to 3 weeks, suppression of elongation has proved to be crucial to survival, or at least to obtain acceptable yields. In the rest of this review, we first discuss the mechanisms of elongation (Sect. 4), followed by the case of deepwater rice which requires elongation (Sect. 5), and then transient complete submergence which requires quiescence (Sect. 6). With these transient floods, favourable underwater gas exchange is likely to prolong survival which may eventually account for differences in performance for variable floodwaters at different locations and times, varying so much in level of irradiance, CO_2 and turbulence.

4 Responding to Rising Deep Water: Rapid Elongation of Internodes

Rice has evolved diverse mechanisms to tolerate inundation, including the internal ventilation strategies described in our partner review (Kirk et al. 2014) and by Nishiuchi et al. (2012). Because oxygenic breakdown of carbohydrates to CO_2 yields energy so abundantly, there has been selective pressure for mechanisms enabling the acquisition of gaseous O_2 in submerged organs. Accordingly, in rising floodwaters the submerged internodes of deepwater rice respond by rapid elongation so as to maintain atmospheric contact of the upper leaves. Internode elongation of deepwater rice is therefore critical to survival in the rising floodwaters in which these extraordinary varieties are cultivated, often in metres of water; the stems are tolerant to submergence while ever the tops remain emergent (Catling 1992; see also Sect. 5). Submerged internodes generally have a supply of O_2 through emergent foliage exchanging gases with stem aerenchyma. Roots situated in anaerobic soil are probably more energetically compromised than the shoots, or than aquatic roots in the water column, because the sediment roots are located in anoxic soil and remote from water-borne O_2 or atmospheric O_2 sources.

Organs from other wetland species share the capacity, like rice (e.g. reviewed by Sauter 2000), to survive inundation through elongation of shoot tissues under water. For example, petioles of *Rumex palustris* (Peeters et al. 2002) elongate preferentially in response to inundation in oxygenated water; low O_2 levels are not required to achieve acclimation. Far fewer wetland/semi-aquatic plants can survive anoxia;

however, rice itself is renowned for the anoxia tolerance of the coleoptile during germination and early growth (e.g. Ishizawa et al. 1999). Extreme examples of anoxia tolerance are growth of the youngest shoots of the aquatic species *Potamogeton distinctus* and *Sagittaria pygmaea*, even being stimulated by anoxia (Ishizawa et al. 1999). Returning to deepwater rice, internodes have attracted the curiosity of researchers as a model organ for studies of rapid plant growth (Kende et al. 1998). Moreover, variation in internodal elongation has been exploited to identify several QTLs for this trait in deepwater rice (Nagai et al. 2012), the most significant example being the two *Snorkel* QTLs on chromosome 12 (Nagai et al. 2010).

4.1 *Internode Morphology and Influence of Submergence on Zones of Cell Division and Enlargement: Features Contributing to Submergence 'Escape'*

Internodes of deepwater rice are adapted to outgrow submergence while relying upon carbohydrates from storage and photosynthesis (Raskin and Kende 1984) and provide a long-distance connection to the atmosphere surrounding the emergent leaves (Kende et al. 1998). Internodes are hollow, making a rigid but economic structure adapted for elongation and internal ventilation. Internodes contain an intercalary meristem (Bleecker et al. 1986) subtending an elongation zone and vascularised, mature, elongated cells which make up the majority of the internode length. A single internode can grow at 5 cm day^{-1} (Kende et al. 1998) and individual internodes can reach lengths of almost 90 cm (Keith et al. 1986), achieved via production of >750 internode cells per file each 2 days in submerged stems, which then elongate prior to maturity (Bleecker et al. 1987). Engagement of several internodes resulted in an overall rate of stem elongation of about 25 cm day^{-1} in deepwater rice (Vergara et al. 1976; Catling 1992). Metraux and Kende (1983) reported that whole plants of the deepwater rice variety Habiganj Aman II elongated to 140 cm over 50 days of submergence, and some varieties in some locations are even more impressive, reaching lengths of up to 7 m (Kende et al. 1998).

Elongation of internodes becomes faster under water than in air (Metraux and Kende 1983). Internode elongation depends upon meristematic activity for a supply of cells that then expand; together these processes sustain extension of the stem. Activity of the 2–3 mm long intercalary meristem of deepwater rice was enhanced by faster cell division under submergence (Bleecker et al. 1987; Metraux and Kende 1984). Such accelerated rates of cell division occur in tissues which are possibly hypoxic but nonetheless with steady O_2 supply through the stem lacunae (large gas channels that enable internal aeration, also called aerenchyma). In addition, the zone of elongation adjacent to the intercalary meristem increases from a 5 mm long segment of tissue to at least 15 mm in length. Concurrent

increases in cortical cell length occurred, with cells up to three times longer (reaching 160 μm) than in stems that were not submerged (Bleecker et al. 1987). The mechanisms that regulate changes in the dimensions of elongation zones and cell size in response to abiotic stresses are not understood and yet these are central to how rapid elongation is achieved in aquatic environments. In submerged rice internodes, a developmental switch to large cells and long meristems makes an intriguing contrast with the diminished growth zone (Liang et al. 1997) and cell sizes (Fraser et al. 1990) observed in maize roots experiencing drought stress, emphasising the strong modulation of growth zones by environment.

Wall-yielding properties were the primary factor enabling rapid elongation under submergence, with a small fall in solute concentrations (i.e. decline in cellular osmotic pressure) more than compensated by looser cell walls (Kutschera and Kende 1988). Hydraulic conductance was not affected by submergence. Accelerated elongation must, however, be sustained by resource supply from any stored reserves and photosynthesis by leaves. Incorporation of ^{14}C into the elongating zone of internodes increased at least an order of magnitude when submerged (Raskin and Kende 1984). Submerged internodes showed a 26-fold increase in import of newly fixed assimilate from emergent leaves, to sustain stem growth (Raskin and Kende 1984).

4.2 The Roles of Dissolved Gases, Hormone Physiology, and Associated Genes in the Submergence Growth Response of Internodes

When immersed in water, plant tissues typically become depleted in O_2 but enriched in CO_2 and ethylene, triggering a complex set of physiological responses that in several wetland species can lead to accelerated shoot elongation. Internodes of rice have evolved molecular responses to dissolved gases that enable them to sense and acclimate to submergence (Rzewuski and Sauter 2008). Ethylene production is sometimes, but not always, stimulated in hypoxic tissue (Raskin and Kende 1984), and since submergence results in entrapment of ethylene, tissue concentrations typically increase even if the rate of production is not stimulated (Voesenek et al. 2006; Jackson 2008). Endogenous ethylene levels accumulated to about 1 ppm in internodal lacunae after 3 days of submergence, coupled with low O_2 (5 %) and elevated CO_2 (4 %) when internodes were in darkness (Stünzi and Kende 1989). Setter et al. (1987a) reported a strong decline in internodal O_2 concentration from the surface to a depth of 1.8 m, and an increase in CO_2 over the same range, in deepwater rice in Thailand. Internodes exposed to an artificial mixture comprising 3 % O_2, 6 % CO_2, and 1 ppm ethylene grew one to two orders of magnitude longer than those in humid air (Raskin and Kende 1984). Illuminated, attached leaves were also important for internode elongation in submerged

deepwater rice, presumably owing to photosynthesis providing O_2, and sugars, to the internodes.

Studies of internodes of deepwater rice have provided insights into the interplay between plant hormones that lead to heightened elongation under water. The caveat is that these phenomena might only apply to deepwater rice internodes because it is clear that genotype and stage of development play critical roles in submergence responses (Keith et al. 1986; Dubois et al. 2011). Rice internodes respond strongly to ethylene, with submergence causing endogenous ethylene to accumulate 50-fold in internodes, where it stimulates elongation, and inhibitors of ethylene such as norbornadiene block elongation (Metraux and Kende 1983; Bleecker et al. 1987). Exogenous ethylene caused non-submerged internodes to elongate as though they were submerged, contrary to the reputation ethylene had gained in early literature as an inhibitor of shoot elongation (Jackson 2008). However, the powerful effects of ethylene only give the illusion of a complete story; Kende et al. (1998) noted that gibberellic acid and abscisic acid also play regulatory roles in internode growth. In evolutionary terms, engagement of multiple hormones in elongation might appear redundant when ethylene is itself so efficacious, but a complex of signalling and downstream gene expression will be outlined below. Ecologically, one could hypothesise that the metabolic investment that directs internodes to elongate by up to 5 cm day^{-1} is so risky as to select for sophistication in the control system. Accordingly, gibberellic acid is a powerful agent in elongating internodes, causing rapid extension, especially when ethylene was present (Raskin and Kende 1984). However, exogenous gibberellic acid can also act independently on internode elongation (Bleecker et al. 1987) and cellulose microfibril orientation (Sauter et al. 1993), suggesting that it is the primary agent for rapid elongation. Interestingly, ethylene not only stimulated endogenous levels of gibberellic acid fourfold but also suppressed abscisic acid levels by 75 % within 3 h, indicating an ethylene-mediated change in the antagonism between gibberellic acid and abscisic acid (Hoffmann-Benning and Kende 1992).

Rice internodes are a model system that has provided many important insights into the molecular basis of hormone action. An early revelation was that submergence caused genes typically associated with hormone metabolism and rapid growth to be expressed. For example, Cohen and Kende (1987) reported that increased activity of ACC synthase, a key enzyme in the ethylene biosynthetic pathway, was a primary event in internodes of deepwater rice, leading to induction of ethylene synthesis within 2 h of flooding (Metraux and Kende 1983). Moreover, Mekhedov and Kende (1996) later noted increased ACC oxidase transcript and enzyme activity in submerged internodes, a process that appears to be regulated by crosstalk between auxin and ethylene action (Chae et al. 2000). The effects of submergence on many genes involved in hormone biosynthesis and signalling remain unknown, especially in gibberellic acid metabolism. A major breakthrough was the identification of two *SNORKEL* genes (*SNORKEL1* and *SNORKEL2*) by Hattori et al. (2009) using positional cloning of QTLs identified from a mapping population (deepwater × non-deepwater rice crosses). Both are ERF transcriptional regulators that control internode elongation by transduction of the ethylene

response to gibberellin-induced elongation. Subsequent gain-of-function analysis was performed by pyramiding three QTLs into a non-deepwater rice to confer the internode-elongation phenotype. Wild relatives of rice will play a role in identifying more key genes in submergence tolerance (see Hattori et al. 2009).

Apart from effects of submergence on genes that encode enzymes in hormone biosynthesis, there are direct effects on the production of new cells and their subsequent expansion. Key genes with products involved in cell division are upregulated by both gibberellic acid and submergence, including a p34cdc2-like histone H1 kinase gene and its corresponding enzyme (Sauter et al. 1995) and histone H3 genes (Lorbiecke and Sauter 1998). Similarly, expression of cyclins and other genes has been invoked as evidence that gibberellic acid enhances meristematic activity, but it remains unclear as to whether this is a direct effect, as proposed by Lorbiecke and Sauter (1998), or the indirect consequence of gibberellic acid-stimulated cell elongation as proposed by Sauter and Kende (1992a). These findings show that induction of the transition from interphase to DNA synthesis (G1 → S) is a critical event that is stimulated by submergence and that entry into the mitotic phase is also accelerated (Kende et al. 1998). Regardless of where the primary effect of increased gibberellic acid during submergence is targeted, as discussed below the effects are not only on genes involved in the cell cycle.

Genes that play a part in the rate of cell elongation, permitting rapid internode extension, are also directly affected by submergence and gibberellic acid. In general, the genes that have been investigated are known to be associated with changes in cell wall rheology in submerged internodes (Kutschera and Kende 1988), with a focus on wall-loosening factors such as enzymes and auxin-induced acidification. Specifically, increased expression of the expansin gene, *OsEXP4*, in the growing zones of submerged or gibberellic acid-treated internodes indicates a mechanistic link between hormones and cell wall behaviour. In addition, these same tissues were susceptible to acid-induced wall extension. Furthermore, Uozu et al. (2000) identified four xyloglucan endo-transglycosylases (XET) from internodes as candidates for wall-loosening effects; these would putatively modify xyloglucan chains in the expanding cell wall. Two of these genes, *OsXTR1* and *OsXTR3*, were preferentially expressed in the elongating zone of internodes and were responsive to additions of gibberellic acid, making them potential candidates for a wall-loosening role. However, invoking expansins and XETs is unlikely to represent the full story of how gibberellic acid leads to longitudinal wall extension. Other factors are conceivably also involved in wall-loosening, e.g. brassinosteroids (Uozu et al. 2000). Much remains to be discovered about the wall-loosening events that must accompany rapid elongation, such as the importance of various cell wall components, e.g. such as β-glucans (Sauter and Kende 1992b). Indeed, identifying the events that lead cell walls to yield to internal pressure has remained a devilishly complex task and the rice internode system, because of its well-described hormonal regulation and rapid growth, is likely to provide further insights.

The restriction of internodes to axial growth is critical to the escape mechanism that allows deepwater rice to survive flooding. At its heart, this phenomenon is the result of the orientation of cellulose microfibrils, which lie transverse to the axis of

growth prior to elongation, become oblique during elongation, and finally re-orient into longitudinal orientations as elongation ceases. Sauter et al. (1993) showed that the longer zone of elongation in internodes treated with gibberellic acid, increasing from 5 to 35 mm, was accompanied by a longer period during which cellulose microfibrils remained in the oblique position. The widely held view that cortical microtubules interact with cellulose microfibrils to dictate the direction of growth was supported for internodes (Sauter et al. 1993). By implication, this is a downstream effect of the increased tissue concentrations of gibberellic acid that accompanies submergence; Sauter and colleagues suggested that events determining microfibril orientation in the meristem dictate gibberellic acid sensitivity in the elongation phase that follows. Furthermore, the maintenance of turgor pressure in submerged internodes (Kutschera and Kende 1988) shows that in spite of very rapid tissue expansion, ion/solute transporters can sustain adequate osmotic pressure in cells. The efficiency of ion transporters in these tissues, and their operation as related to tissue O_2 regime, deserves attention.

Differentiation of the internode is also important, both because it dictates the time at which elongation must cease and also because it will influence the strength of the stems. While deepwater rice is supported largely by the water in which it grows, and indeed often harvested *in situ*, if waters recede then 'kneeing' and lodging resistance to maintain the panicle off the ground are vital for yield (Catling 1992). Bleecker et al. (1987) showed that xylem vessel development on the distal margins of the zone of elongation was delayed in submerged internodes, with secondary wall thickening and end-wall formation inhibited. This was followed by the observation that the activities of coniferyl alcohol dehydrogenase (CAD) and phenylalanine ammonia-lyase (PAL) in rapidly elongating internodes were reduced to one-sixth of the control rates (Sauter and Kende 1992b), strongly indicating that fast growth has a penalty of slower cell wall lignification. There is a place for a more complete analysis of differentiation and secondary wall formation in deepwater rice internodes, and again, their rapid elongation and spatial separation of the developing tissues might constitute a fine model for studies of cell wall biochemistry.

4.3 Summary

Internodes of rice are a valuable model for the study of plant growth under water and more generally. In general, submerged tissues are characterised by diminished O_2 levels and accumulation of CO_2 (both at night) and with increased ethylene. Internodes have evolved a complex set of perception and signalling pathways for metabolic responses that allow rapid elongation to develop canopies with foliage held above the floodwater. Ethylene signalling, resulting in increased gibberellic acid in the downstream regulatory cascade, sets off pathways of gene expression and cell wall changes that allow overall stem extension rates of up to 25 cm day^{-1}. Research to date has concentrated on genes involved in mitosis and cell wall

loosening enzymes such as expansins, but far more genes that connect signalling with the mechanics of growth remain to be discovered (Vriezen et al. 2003). Less is known about how submergence affects later differentiation in internodes.

5 Acclimation–Adaptation to Long-Term Flooding

'As the water rises the rice will follow' Thai colleague

As stated before, for long-term flooding lasting several months, elongation is indispensable to ensure an adequate emergent canopy. Nearly all rice genotypes respond to flooding by elongation of shoot organs, be it with extension of leaf sheaths and to a lesser extent leaf blades, or by internode elongation, which is the main elongation in very deep water >1.5 m. Mechanisms of internode elongation were described in Sect. 4; importantly, many lowland rice genotypes respond to flooding by substantial leaf blade and sheath elongation, rather than by internode elongation (Sect. 5.1).

In this section we address the physiological consequences of the elongation of internodes and sheaths triggered by submergence, and also consider the functioning of aquatic roots. We will not cover other desirable traits for tolerance of long-term flooding, such as ability to tiller under water and 'kneeing ability' (Catling 1992). Elongation maintains a portion of foliage above water, in particular very important during the reproductive phase, i.e. during panicle formation. The adaptation of deepwater rice to long-term flooding causing partial submergence is clearly demonstrated by reasonable grain yields of up to 2.5 t ha^{-1} at water depths of 0.9–2.1 m and even in 3.5 m of water some varieties yield 2 t ha^{-1} (Catling 1992).

5.1 Internode Versus Leaf Elongation

In deepwater rice, elongation is predominately by internodes but leaves (mainly the sheath) also elongate. This leaf elongation is triggered by ethylene, similar to that described in Sect. 4 for internodes. For example, with shoots of IR42 in a gas phase containing ethylene at 0.35 Pa leaves became 1.6 times longer than those in the absence of ethylene (Jackson et al. 1987). In the case of IR42 this elongation is disastrous during complete submergence by transient floods (Sect. 6); this variety is commonly used as a sensitive check in experiments on complete submergence. By contrast to lowland rainfed rice varieties exposed to transient complete submergence, sheath elongation would be of benefit to tall genotypes exposed to prolonged moderate floods, so as to maintain a substantial shoot portion above water.

A key investigation on internode elongation in response to rising water used a set of recombinant inbred lines derived from crosses between the semi-dwarf IR74 and Jalmagna, a traditional tall deepwater rice landrace (Sripongpangkul et al. 2000). Major QTLs associated with internode elongation were located on chromosomes

1 and 4, and an epistatic QTL on chromosome 5. During phenotyping, waters were raised at 5 cm day^{-1} till they reached 1.2 m and then were maintained for 7 days (Sripongpangkul et al. 2000). At the end of these 7 days, height for Jalmagna was 1.5 m and that of IR74 was 0.78 m; i.e. IR74 was completely submerged for at least the last 8 days. In the deepwater variety, internode length increased by 105 cm, while there was no internode elongation in IR74. Yet, in clear water IR74 also survived, which might have been due to leaf elongation and/or the ability to photosynthesise under water. QTL expression depended on irradiance in the waters, with the expression of QTLs associated with internode elongation increasing in muddy water, but QTLs associated with leaf elongation expressed better in clear water (Sripongpangkul et al. 2000). This interesting experiment illustrates the importance of gene × environment interactions on flooding tolerance in rice. A caveat is needed. The study was based on only one recombinant inbred population, so the detected QTLs may only relate to this particular population and phenotyping conditions. In view of the importance of the demonstrated plasticity depending on illumination, a key characteristic of flooded environments, confirmation in other populations would be worthwhile, as would further fine-mapping and candidate gene identification.

5.2 *Consequences of Elongation for Photosynthesis and Carbohydrate Balance*

The investment of carbohydrates in elongation may incur a penalty of lower grain yield, as supported by a substantial reduction in harvest index (grain/total shoot weight), and in yield as water depth increases (Fig. 4). Harvest indices were 0.08–0.34 in seven deepwater rice varieties compared to about 0.55 in modern varieties in paddy conditions (Catling 1992). Interestingly, harvest indices of the two systems converge when the grain mass is expressed as a proportion of dry mass above the water (Catling 1992). So, in consideration of the carbon used to construct and maintain the underwater portions, either the submerged part of the foliage produced adequate carbohydrates to sustain itself, or the photosynthetic efficiency of the emergent foliage was high. It therefore seems likely that adaptations that enhance photosynthesis, in either or both the emergent and submerged foliage portions, might improve yields.

Photosynthesis of submerged leaves depends upon light intensity in the water and availability of CO_2 (Sect. 3). These factors are usually well below optimum, while the situation is further aggravated by premature senescence of submerged leaves (Ella et al. (2003a) for rice, Mauchamp et al. (2001) for *Phragmites australis*). These penalties can be partly compensated for by increased photosynthesis by the emergent foliage, as shown by a threefold increase over the non-submerged control, using isolated internodes with a 70 cm long leaf attached (Raskin and Kende 1984). Similar, but much less dramatic, responses were found

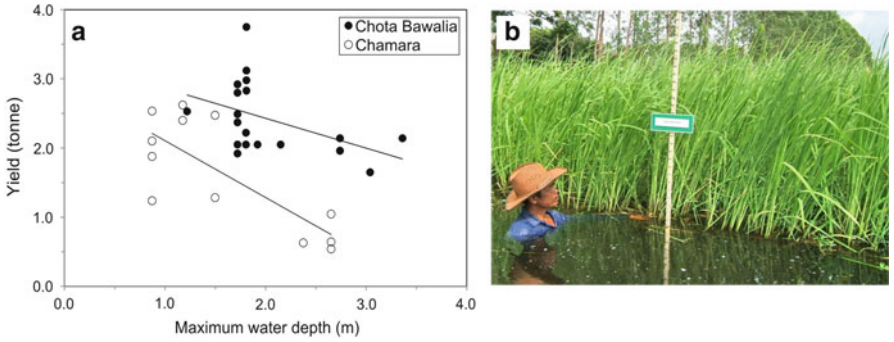

Fig. 4 (**a**) Grain yield of deepwater rice decreased with increasing maximum water depth in farmer's fields in Bangladesh (Catling 1992). This decrease is associated with a reduction in harvest index (grain weight/total shoot weight) (Catling 1992). However, when the harvest index is expressed on the weight of the emergent foliage this index is sometimes even higher than when the whole shoot is in air (Catling 1992). (**b**) Photograph of deepwater rice grown at the Prachinburi Rice Research Station, Thailand, kindly provided by Ms Udompan Promnart. Part A is from Catling (1992), *Rice in Deep Water* (First Edition), with permission from The Macmillan Press London and Basingstoke

when 50 % of the canopy of rice was submerged; net assimilation rate in the emergent leaves was 20 % higher than in comparable leaves of plants which were not submerged [Sakagami et al. (2009) for rice; for similar data in *Phragmites australis*, see Mauchamp et al. (2001)].

Carbohydrate translocation to the elongating zone and lengthening internodes are both greatly accelerated during partial submergence, as would be expected. In deepwater rice after 3 days of partial submergence, the internodes existing before partial submergence had lost 65 % of their starch and had a 70-fold increase in amylotic activity, while translocation of recently fixed ^{14}C by the emergent foliage to submerged tissues increased 26-fold (Raskin and Kende 1984).

5.3 *Sediment and Aquatic Roots*

Anecdotal evidence indicates that for rice in deep water many of the sediment roots decay (Bleecker et al. 1987; Suge 1985) at least after several months of flooding (Catling 1992). Suggestions that these roots die due to anoxia are plausible because of the increased length of the path for O_2 diffusion, and in the anaerobic soil typically found around rice roots, radial O_2 losses to microbial and other oxidative reactions would further compete for O_2 with distal root apices (Kirk et al. 2014). However, there has been no evaluation of O_2 deficiency in distal root apices; O_2 deficiency in these roots would be particularly likely in cases where the ligule of the uppermost leaf was submerged (Pearson and Jacobs 1984). An alternative is that the

strong diversion of carbohydrates to the elongating internodes would lead to carbohydrate starvation in the roots.

Aquatic roots may supplement or replace inefficient or decaying sediment roots. In three varieties of deepwater rice, there were 12–26 aquatic roots per node (Inouye and Mochizuki 1980) and aquatic roots reached an average length of 15 cm (Catling 1992). The initiation and development of these roots are determined by hormones. When an excised 25 cm long stem section was exposed to 1 ppm ethylene and 0.1 ppm gibberellic acid, its node developed 11 adventitious roots up to 18 mm long within 48 h exposure; stem sections without the exogenous hormone failed to develop these roots (Suge 1985). Subsequent studies showed that ethylene was the primary signal inducing the outgrowth of adventitious roots from stem nodes (Lorbiecke and Sauter 1999), including interactions with gibberellic acid and abscisic acid (Steffens et al. 2006) and also involving death of epidermal cells overlaying root primordia so as to facilitate root emergence (Steffens and Sauter 2005).

In the absence of further detailed studies on aquatic roots of rice, we discuss a quantitative study on the marsh species *Cotula coronopifolia* and *Meionectes brownii* (Rich et al. 2012). Submergence of one-third of the shoot for 28 days, with the sediment roots in 0.1 % stagnant agar nutrient solution, stimulated aquatic roots so that they contributed approximately 25 % of total dry weight of the plants, with individual axes of aquatic roots up to 26–50 cm (Rich et al. 2012). The aquatic roots contributed 90 % to the sum of aquatic and sediment root dry weight, the latter becoming flaccid and presumably decaying (Rich et al. 2012). Whether such trends would be found for deepwater rice needs to be established.

Aquatic roots may contribute to mineral nutrition of submerged plants especially for species with gas films on submerged foliage (Sect. 3; Fig. 2c) which would prevent direct nutrient absorption by these shoot tissues from the floodwater. Contributions to nutrition by aquatic roots would depend on several factors. Nutrient uptake would be limited by low concentrations in the floodwater and the impact of unstirred boundary layers. In a deepwater rice field in Thailand, K^+ was 0.07–0.11 mM and N was 0.02–0.14 mM (Setter et al. 1987b). In turbulent solutions, the upper end of these ranges would give about half of the maximum uptake rate by the seminal roots of cereals (Epstein 1972). Absorption of $^{15}NH_4^+$, supplied to aquatic roots for 10 days at 3.5 mM, contributed 50 % to the total N uptake of the culm and 75 % of the N content in aquatic roots (Khan et al. 1982). One reason for this high % might be that the total N supplied to the aquatic roots was at the high 3.5 mM. All the same, effects of the low nutrient concentrations in the floodwater are likely to be mitigated by the large pool of nutrients in the bulk water, which is often streaming, thereby reducing the diffusion limitation (i.e. narrower boundary layers) between floodwater and plants.

The O_2 status of aquatic roots, and thus energy available for ion transport, should be adequate as aquatic roots contain aerenchyma with O_2 transport via the internodes complemented by some *in situ* O_2 evolution by photosynthesis in these roots that are likely to contain chloroplasts (Sect. 3.4), as well as O_2 entry directly from the floodwater into aquatic roots. In illuminated adventitious roots,

chloroplasts are found in inner and outer cortex in non-aerenchymatous tissue and even in the radial cell strands between the lacunae (Armstrong et al. 1994). Aquatic roots of rice have well-developed aerenchyma and cubic cell packing in the cortex (Inouye and Mochizuki 1980), features that enhance internal aeration (Kirk et al. 2014).

5.4 Summary

Insight into the mechanisms of acclimation and adaptation to long-term flooding will require time sequences of rates of photosynthesis of emergent and submerged leaves, internode elongation, and carbohydrate levels in various plant parts. One key question is whether grain yield is restricted by inadequate carbohydrates. That presumably depends on the proportion of foliage that emerges above the floodwater and the efficiency of photosynthesis in both emergent and submerged leaves. Information on carbohydrate levels in internodes and leaves during grain filling is required.

Other important questions are whether the aerenchymatous sediment roots of rice become O_2 deficient in these deeper floods because the diffusion path along the shoots is considerably lengthened. There are as yet no data on O_2 concentrations and possible anaerobic catabolism, which are important to establish the cause of likely inefficient sediment root functioning and decay. Finally, the significance of the aquatic roots remains to be elucidated; i.e. do these roots contribute substantially to the mineral nutrition of deepwater rice? In particular, studies are required on their ion uptake relative to the sediment roots, at ion concentrations and other conditions (e.g. O_2 levels) relevant to the field.

6 Transient Complete Submergence

In this section we discuss the often disastrous consequences of elongation during transient complete submergence, sharply contrasting with the beneficial effects of elongation during long-term flooding, discussed in Sect. 5. This contrast poses a dilemma, since in regions with long-term flooding, complete and partial submergence are commonly experienced during the same season. The dilemma was illustrated during flooding with a constant 0.5 m water depth for 65 days of a short variety (cv. Swarna) containing the *SUB1* QTL (cv. Swarna-Sub1), which resulted in yield being reduced by 60 % (Singh et al. 2011). Water depths were increased only by 0.03–0.05 m day^{-1} to avoid complete submergence (Singh et al. 2011), and the proportion of emergent canopy was not recorded. In contrast, FR13A and its derivative IR497830 were both reduced in yield by only about 35 %; both these two genotypes contain the *SUB1* QTL; however, they are inherently taller and have greater capabilities for some elongation than the dwarf Swarna-Sub1 (Singh

et al. 2011). Thus, during these long-term floods starting early after seedling establishment, a combination of the *SUB1* QTL and inherent tallness resulted in the best performance (Singh et al. 2011).

In about 50 % of rainfed lowland ecosystems the shoots of rice are sometimes submerged during transient floods most commonly lasting a few days to 2 weeks. Such flooding imposes a major constraint to rice production in 12.5 % of the world's rice growing areas (Jackson and Ram 2003). During this flooding, reduced irradiance and severely limited exchange of O_2 and CO_2 between floodwaters and shoots (Sects. 2 and 3) are added to the challenge of coping with the anoxic soils (Kirk et al. 2014). Tolerance to this challenging environment is summarised in Fig. 5, which is based on comparisons between submergence-tolerant and submergence-intolerant genotypes under the usual field conditions of strongly reduced irradiance and CO_2 fluxes into the leaves. It is important to note that the syndrome in the intolerant genotype does not apply when there is substantial underwater photosynthesis. In the earlier described CO_2 enrichment experiments, one set of plants of an intolerant variety was completely submerged for 84 days, with a combination of 1 kPa CO_2, vigorous turbulence, and high light intensity; then the dry weight of the shoots reached 62 % of the plants with shoots in air (Setter et al. 1989).

6.1 A Summary of the Genetic Basis of Submergence Tolerance: SUB1

Large genotypic differences in submergence tolerance have been known for decades, as revealed in Asia-wide screening programmes by IRRI. This research began in the 1970s when landraces tolerant to submergence, such as FR13A, were discovered (Palada and Vergara 1972; Khush and Coffman 1977). Tolerance to submergence during the vegetative phase within *Oryza sativa* is rare; only 2 % of approximately 18,000 genotypes showed tolerance (Setter and Laureles 1996). The IRRI programmes were based on conventional plant breeding, which by the 1990s had been successful in developing submergence-tolerant dwarf varieties; however, these varieties still had several undesirable characteristics inherited from the donor parents such as FR13A (Mackill et al. 1993). Subsequently, QTL analyses, molecular marker development, and marker-assisted selection were used to identify and introgress the *SUB1* QTL, which controls up to 70 % of the submergence tolerance of FR13A, into the background of popular rice varieties in Asia (Toojinda et al. 2003; Xu et al. 2006; Mackill et al. 2012; Ismail et al. 2013).

Tolerance to transient submergence in the field, and in systems mimicking field conditions (i.e. relevant CO_2 levels and low irradiance), appears related to suppressed response to ethylene, which in turn distinguished three characteristics: reduced shoot elongation, less carbohydrate consumption, and slower chlorophyll degradation (Sects. 6.2.1–6.2.3). Other less well-established effects include

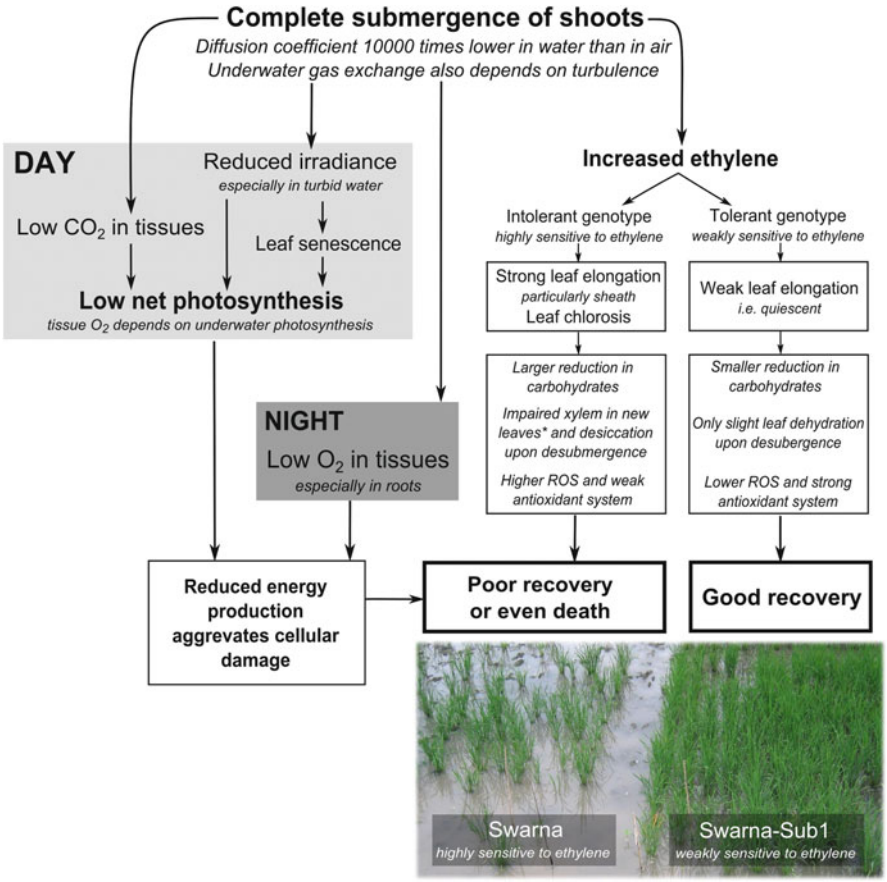

Fig. 5 Responses of rice to transient complete submergence lasting a few days up to 3 weeks. The response will vary in degree dependent on the prevailing irradiance, turbulence, and CO_2 concentration in the floodwater. In the field, CO_2 is commonly above air-equilibrium values, but it would be unusual to have high turbulence and high irradiance in floodwaters. The dependence of responses on these environmental conditions has been demonstrated in controlled experiments at 1 mM CO_2 with high turbulent mixing from gas bubbling and high light; then even an intolerant variety grew well when submerged for 60 days (Setter et al. 1989). Very slow leaf elongation is aptly called a 'quiescence response' (Bailey-Serres and Voesenek 2008); the matching term for the alternative 'escape response' is not used here since the consequence of strong elongation under complete submergence is severe injury and/or death. Note that formation of free radicals of oxygen is usually a consequence of injury due to other causes, but these radicals can severely aggravate injury, so efficient scavenging systems will often substantially aid recovery after desubmergence. The photograph insert shows sensitive (Swarna) and tolerant (Swarna-Sub1) after being subjected to 17 days of submergence in IRRI field ponds (photograph taken 1 month after desubmergence). *Asterisk*: the impaired xylem only applies to elongation, not to chlorosis

(1) tolerance to hypoxia-anoxia (Sect. 6.2.4), (2) mitigation of water deficits, which occur after desubmergence, even though roots remain in flooded soil (Sect. 6.3.1), and (3) induction of enzymes involved in scavenging free radicals of O_2 (Sect. 6.3.2).

Remarkably, many of the acclimations to the key factors listed above and shown in Fig. 5 are regulated by *SUB1A*, a member of the ERF family. This gene is present in most *Indica* rices, but absent in *Japonica* rices and from the tested wild *Oryza* species (Bailey-Serres et al. 2010). A one point mutation resulted in two alleles of *SUB1A* gene, *SUB1A-1* specific to tolerant genotypes, and *SUB1A-2* which is present in most *Indica* genotypes and results in a sensitive phenotype. *SUB1A-1* suppresses ethylene responsiveness and may also regulate downstream expression of other genes endowing tolerance to the adverse consequences of submergence (Jung et al. 2010). Introgression of *SUB1A-1* (by its presence in the *SUB1* QTL) into modern varieties has increased submergence tolerance by 1–2 weeks and also provided elite Sub1 breeding lines, and commercial releases (called Sub1 varieties) for some regions in Asia (see Fig. 5 for field response; extensive documentation in Singh et al. 2009; Xu et al. 2006; Bailey-Serres et al. 2010; Mackill et al. 2012; Ismail et al. 2013). The benefit of the *SUB1* QTL for yield after submergence depends upon the duration of the submergence and water characteristics (Singh et al. 2009; Sarkar et al. 2009), influencing survival and recovery to enable good plant density resulting in yield improvements of 1.7–3.8 t ha^{-1} above the modern variety into which it was introgressed (Mackill et al. 2012). Tolerance of Sub1 lines/varieties was superior whether complete submergence was imposed on 7-day-old seedlings or at any later stages up to about 1 month before flowering, and works in different genetic backgrounds and environments (Mackill et al. 2012). That validity is fortuitous, since the standard IRRI screening trials developed decades ago have always started the submergence when seedlings were 10–14 days old (Palada and Vergara 1972). The genotypes differ in degree, not in kind, and as emphasised by Jackson and Ram (2003), even FR13A suffers when the 'stress' becomes severe.

The successful deployment of the *SUB1* QTL in rice breeding vindicates the statement of Jackson and Ram (2003) that the research on submergence tolerance of rice has met the 'major challenge of harnessing molecular biology (i.e. molecular breeding) to ameliorate the effects of an environmental stress'. In other words, there has been vindication of the success of a dynamic interaction between disciplines of different levels of complexity (or organisation), as phrased eloquently by Crick (1994). In this section, we give for completeness a brief description of the discovery and deployment of the *SUB1* QTL (for detailed accounts, see Bailey-Serres et al. 2010; Mackill et al. 2012; Ismail et al. 2013) and then critically review the physiological evidence on the relative importance of the various factors known to be involved in submergence tolerance of rice, as influenced by the *SUB1* QTL. As stated in the review by Jackson and Ram (2003), the challenge remains to assess the importance of each physiological process for submergence tolerance across diverse environments and durations of submergence.

Studies on the genetic basis for submergence tolerance in rice found a major QTL located on chromosome 9, named *SUB1* (Xu and Mackill 1996; Xu et al.

2000). These studies used a population developed from a cross between the tolerant *Indica* line IR40931-26, a derivative of FR13A, and a flooding-intolerant *Japonica* line, PI543851 (Mackill et al. 1993). The importance of the identified major QTL, *SUB1*, was confirmed in a subsequent study (Nandi et al. 1997). This QTL accounted for 35–70 % of the phenotypic variation in submergence tolerance in various populations (Fukao et al. 2006). The large contribution of the same QTL on chromosome 9 was further confirmed in three doubled haploid mapping populations obtained from crosses between submergence-tolerant and submergence-intolerant parents (Toojinda et al. 2003). Significantly, these studies showed that traits such as early senescence and elongation were not independent, instead appearing to be under the control of the same QTL on chromosome 9 (Toojinda et al. 2003).

The *SUB1* QTL was shown to contain three genes encoding ERFs, namely *SUB1A*, *SUB1B*, and *SUB1C*. Of these three, only *SUB1A* confers submergence tolerance and this gene is missing in genotypes which are intolerant to submergence (as autotrophic seedlings) (Xu et al. 2006; Fukao et al. 2006; Bailey-Serres et al. 2010). Two alleles of *SUB1A* were found: *SUB1A-1*, which endows tolerance, and *SUB1A-2*, which does not. The two alleles differ by one amino acid substitution at position 186, where proline in *SUB1A-2* is replaced by serine in *SUB1A-1*. The *SUB1A-1* allele dampens ethylene production and responsiveness, and limits ethylene-induced gibberellic acid-promoted shoot elongation (Fukao et al. 2006; Xu et al. 2006; Bailey-Serres et al. 2010). Introgression of the *SUB1* QTL, through marker-assisted selection of backcross populations, endowed tolerance to submergence in the otherwise intolerant *Indica* variety Swarna, while maintaining otherwise desirable characteristics of this widely grown Indian variety (Xu et al. 2006; Fukao et al. 2006; Bailey-Serres et al. 2010). The resulting submergence-tolerant selection was named Swarna-Sub1 and released to farmers. Similar results have now also been achieved with several modern rice varieties. Critically, in the absence of complete submergence the lines containing the *SUB1* QTL yielded equally to the recipient parent varieties (Xu et al. 2006; Mackill et al. 2012). In addition to suppressing ethylene responsiveness, the *SUB1* QTL influences expression of genes associated with anoxia, drought, and detoxification of reactive oxygen species (ROS) (Fukao et al. 2006, 2011), as discussed in the next sections of this review.

6.2 Responses During Submergence

6.2.1 Ethylene Responsiveness of Shoot Elongation

There is no doubt that blocking the classical ethylene responses such as elongation and chlorosis of sheaths and leaves (Sects. 4 and 5) is a major component of tolerance to transient complete submergence. It is less certain whether this overall effect can always be mainly attributed to avoiding carbohydrate starvation that could result from carbohydrate consumption during extension growth. There are

indications that at times susceptibility to chlorosis Sect. 6.2.3, anoxia Sect. 6.2.4, or water deficits after desubmergence Sect. 6.3.1 may better explain the lack of tolerance, than carbohydrate starvation during submergence Sect. 6.2.2.

The role of ethylene in the shoot elongation response of rice during submergence was shown in glasshouse studies (Jackson et al. 1987). Leaf tissues of submerged rice contained 0.7 Pa ethylene compared with 0.2 Pa in air. Complete submergence for 4 days resulted in 45–65 % increased leaf elongation and 50–75 % decreases in chlorophyll in leaf blades of a submergence-intolerant variety, IR22, but not in two submergence-tolerant landraces, FR13A and Kurkuruppan (Jackson et al. 1987). These different responses of the genotypes were also elicited by exposure to 0.35 Pa ethylene in air but prevented by addition of 1 kPa CO_2, a known antagonist of ethylene action (Jackson et al. 1987). Subsequently, in a key paper, Setter and Laureles (1996) demonstrated that reduced elongation endowed tolerance of submergence: gibberellin application just before 7–10 days submergence enhanced elongation and decreased survival from 85 to 35 % in the submergence-tolerant FR13A, while paclobutrazol, a gibberellin synthesis inhibitor, reduced elongation and increased survival from 0 to 100 % in two intolerant varieties. Similar results were found in experiments by Das et al. (2005).

6.2.2 Carbohydrate Conservation by the 'Quiescence Response'

The response to gibberellins and its inhibitors formed the basis of a useful working hypothesis: elongation in the absence of substantial photosynthesis would exhaust carbohydrate reserves and so jeopardise survival of submerged rice (Setter and Laureles 1996). Conversely, the 'quiescent response' would conserve carbohydrates for catabolism, essential to produce energy for longer-term survival (Setter and Laureles 1996) and subsequent recovery upon desubmergence (Jackson and Ram 2003). This view of carbohydrate starvation during transient submergence as a fatal consequence of increased elongation has been generally accepted (Toojinda et al. 2003; Fukao et al. 2006, reviews by Jackson and Ram 2003; Bailey-Serres and Voesenek 2008; Colmer and Voesenek 2009; Bailey-Serres et al. 2010). Here, we discuss evidence that carbohydrate starvation may, at least sometimes, not be the key factor in determining submergence tolerance.

The first evidence for carbohydrate depletion during complete submergence of rice was based on detailed work by Yamada (1959), as quoted by Jackson and Ram (2003). Subsequently, testing of a range of varieties established high correlation coefficients between concentrations of starch and soluble carbohydrates in the shoots and recovery from submergence (i.e. survival, judged a few weeks after desubmergence), particularly for carbohydrate concentrations at time of desubmergence (Jackson and Ram 2003; Das et al. 2005). Comparisons between the submergence-tolerant FR13A and two submergence-intolerant varieties in two sets of experiments show poor survival of the intolerant varieties even when considerable starch remained (Table 1).

Table 1 % Starch in whole shoots (dry weight basis) and % survival in rice varieties tolerant and intolerant to complete submergence

Variety	% Starch (dry weight basis) at desubmergence	% Survival	Days of submergence	Reference
FR13A (Tol.)	21	75	15	Singh et al. (2001)
FR13A (Tol.)	10	83	10	Das et al. (2005)
IR42253 (Intol.)	9	15	15	Singh et al. (2001)
IR42 (Intol.)	4.2	8	10	Das et al. (2005)

Earlier it was argued that even as little as 5 % starch (dry weight basis) would be sufficient for 190–360 h maintenance respiration (Setter et al. 2010). It was assumed that, if required, all the starch could be hydrolysed (Setter et al. 2010). However, there is doubt whether that assumption holds. In one investigation, the starch of leaves was approximately 5 % in the tolerant genotype at 4 days after submergence and did not decrease over the next 2 days, while in the submergence-intolerant genotype starch only decreased from 2 to 1.3 % (Das et al. 2000); i.e. starch reserves were not entirely consumed. Even so, doubt remains about the putative key role of starch in preventing carbohydrate starvation assumed to contribute to submergence intolerance. There was little survival of the submergence-intolerant IR42 when the starch in shoots at desubmergence was 4 % and in IR42253 it was even 9 % (Table 1), levels which decrease substantially when submergence is prolonged (Das et al. 2000). In addition, the tolerant genotype M202-Sub1 survived for 98 % after 12 days submergence, even though starch during the last 4 days of submergence was steady at only 1 % (Fukao et al. 2006). As far as we know, there is no information on the total reserve carbohydrates in the growing zones; yet this parameter might be critical to survival and particularly to regrowth after desubmergence. So, the question of the putative role of carbohydrate starvation causing death during submergence remains in doubt, and a systematic analysis of the ability of various rice genotypes to utilise starch reserves needs further experimentation.

Summing up, more direct tests of carbohydrate limitation insubmerged rice are required and could be obtained by supplying sugars during either submergence, or after desubmergence. Of course, the addition of sugars during submergence is not trivial since substantial bacterial growth has to be prevented. It would be worthwhile testing for rice the stem infusion approach used by Zinselmeier et al. (1999) in their studies of the influence of stem carbohydrates on kernel filling in maize during water deficits.

6.2.3 Chlorosis and Photosynthesis

Intolerant genotypes usually become chlorotic within a few days of submergence, with evidence that responsiveness to ethylene underpinned genotypic responses of this parameter to submergence. Direct evidence for this proposition was obtained using an inhibitor of ethylene action (MCP) applied during the last 5 h before submergence. Treatment with MCP antagonised ethylene and improved survival of IR42 from 53 to 83 % after 6 days of submergence (Ella et al. 2003a). Consistently, the MCP-treated plants showed delayed depletion of sugars by 15 % and of starch by 20 % (Ella et al. 2003a). Critically in this experiment the MCP treatment did not suppress elongation; hence the increased carbohydrate status and survival were attributed to smaller reductions in chlorosis and chlorophyllase activity (Ella et al. 2003a), and therefore presumably improved underwater photosynthesis (Sect. 3) as well as enhanced photosynthesis during the recovery phase following desubmergence. This finding highlights that elongation by itself is not always fatal, but elongation and chlorosis may act, either in concert or independently, to cause carbohydrate starvation. Confirmation of the observations by Ella et al. (2003a) is required, in particular by evaluation of growth and other physiological characteristics much earlier than 21 days after desubmergence; the latter was used by Ella et al. (2003a).

Submergence appears to damage the photosynthetic apparatus of rice, as demonstrated when photosynthesis was measured immediately following desubmergence (Smith et al. 1988). Leaves of FR13A submerged for 2 days were then able to photosynthesise in air at 45 % of the rate of leaves of plants which had not been submerged. Photosynthesis after return to air by intolerant IR42 was already depressed, after 1 day of submergence, to 40 % of the non-submerged plants (Smith et al. 1988). These data indicate very rapid changes in the photosynthetic apparatus of IR42, presumably distinct from the earlier described chlorosis, since at 1 day of submergence chlorophyll had not yet decreased (Smith et al. 1988).

6.2.4 Importance of Tolerance to Hypoxia-Anoxia

There are a number of indications that anoxia-hypoxia may, at least in some ecological situations, be an important component of the adverse effects of submergence. Evidence for anoxia-hypoxia includes several investigations on metabolism, as well as regulation by *SUB1A-1* of transcripts of genes encoding enzymes known to be involved in anaerobic metabolism (discussed in this section). Yet, other investigators have concluded that the role of anaerobic metabolism is rather minor for submerged rice (Jackson and Ram 2003), although we feel that conclusion may not be correct in several situations used by Jackson and Ram to support their conclusion (see also Appendix). As reported earlier (Sect. 3.3), a recent study in which root O_2 was monitored for submerged rice in the field found very low O_2

concentrations even in the upper parts of roots (Winkel et al. 2013), so anoxia would have been likely in deeper root portions.

The relative importance of anoxia in submerged rice may well depend on factors such as the duration of submergence, irradiance, water temperature, and dissolved O_2 concentrations (Das et al. 2009), to which we add the strength of the biological sink for O_2 in the anoxic soil. A major difficulty in interpretation is that mere evidence for components of anaerobic catabolism gives no guarantee that the tissues suffered O_2 deficiency, since that type of catabolism may also occur in air, when oxidative phosphorylation is inhibited, e.g. due to injury of the mitochondria (reviewed by Gibbs and Greenway 2003). Some key molecular evidence for the possible importance of anaerobic catabolism during submergence is the regulation by *SUB1A-1* of several other genes than those involved in elongation and chlorosis, e.g. genes known to be involved in anaerobic catabolism. In lines with *SUB1A-1*, submergence resulted in the shoots having higher levels of transcripts of alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC), and sucrose synthase (Fukao et al. 2006). Further evidence was equivocal: ethanol formation and maximum catalytic activities of PDC were only 1.35 times higher in the line carrying *SUB1A-1* than in the wild type, with a twofold increase for ADH (Fukao et al. 2006; stagnant solutions, no recovery checks for enzyme assays). For the key enzyme PDC, the total catalytic activities of the submerged leaves were only 0.04–0.06 μmol g^{-1} protein min^{-1}, compared to 0.5–0.7 μmol g^{-1} protein min^{-1} for anoxic rice coleoptiles (Gibbs et al. 2000), perhaps indicating that there was only a small anoxic core (i.e. tissue zone) in the shoots of the submerged seedlings in Fukao et al. (2006); cf. Gibbs and Greenway (2003). Coordinated regulation of genes for ethanol formation and genes limiting elongation is not unexpected; prevention of elongation during submergence *ipso facto* gives a higher risk of O_2 deficit. The data by Fukao et al. (2006) indicate that *SUB1A-1* produces a transcription factor which acts on the anaerobic response element, discussed in detail by Dennis et al. (1991).

Evidence for the importance of ADH during submergence, and therefore by inference of anaerobic catabolism, was obtained in two physiological studies. In a study comparing a wild-type and a *rad* mutant, which has a single recessive mutation with severely reduced ADH1 total catalytic activity, 7-day-old etiolated seedlings[1] were submerged, and after another 7 days the plants were re-aerated and only the wild type resumed extension growth (Matsumura et al. 1998). The *rad* (ADH1) mutant had no detectable ADH in the shoots and only 40 and 60 % of the wild type in the roots and scutellum, respectively (no enzyme recovery checks). In the second investigation the seedlings were 14 days old when the submergence started. There was an impressive correlation between ethanol formation and survival during complete submergence, based on wild-type and transgenic plants, with differences of fourfold in both PDC activity in vitro and ethanol formation (Quimio et al. 2000). The ethanol and PDC were measured in tillers in anoxic agar for 24 h,

[1] That is well past the germination stage.

while the survival was measured for whole plants submerged for 14 days, with survival tested after 5 and 21 days recovery (Quimio et al. 2000). These two sets of data strongly suggest an important role of glycolysis-linked ethanol formation pathway during submergence even in plants well past the coleoptile stage. Importantly, the shoots of the seedlings in these experiments were merely in non-stirred, not in anoxic, solution; hence the results are more relevant to submergence during transient floods than experiments on coleoptiles using anoxia (e.g. Gibbs et al. 2000). For example in Quimio et al. (2000) the seedlings were in light–dark cycles; O_2 in the water at the time of submergence was 0.03 mM and increased after 14 days to 0.17 mM.

Most of the investigations on anoxia-hypoxia in rice during complete submergence have focused on leaves. Yet, the most likely location of O_2 deficiency during complete submergence is in the roots (Waters et al. 1989; Winkel et al. 2013), being furthest from the O_2 supply and surrounded by anoxic soil (Kirk et al. 2014). Even under the rather benign conditions of submergence in solution culture and 10 kPa O_2 flushing around the shoots, the rice roots, which were in non-stirred nutrient solution, produced appreciable ethanol during the night (Waters et al. 1989). Rates were at times as high as 2.3 μmol g^{-1} fresh weight h^{-1} and the ethanol produced was consumed again during the day (Waters et al. 1989). In these experiments, roots ceased elongating during the night, but elongation resumed during the day. In the field, relief of O_2 deficiency during the day might not always occur in view of the low light intensities usually occurring at and below 20 cm from the surface of turbid waters (Ram et al. 1999, Sect. 2.2) and a possible large sink for O_2 in the reduced soil (Kirk et al. 2014). Severe damage to leaves would reduce the O_2 flux to roots derived either from photosynthesis or from the floodwaters, and result in low O_2 concentrations in the roots; i.e. anoxia in the roots cannot anymore be avoided when the O_2 supply from the shoots is diminished.

In conclusion, the importance or otherwise of anaerobic catabolism during complete submergence of rice remains uncertain. The difficulty of establishing whether anoxia in part of the tissue affects the overall plant performance has been discussed in our companion review (Kirk et al. 2014). Whether anaerobic catabolism becomes an important component may well depend on environmental factors in the floodwater. It is worth reiterating that, in view of the paucity of information on the response of the roots to complete submergence of the whole plants, these deserve more attention, since roots in the anoxic soil are the most likely organs to suffer severe O_2 deficiency (Waters et al. 1989; Winkel et al. 2013, Appendix).

6.2.5 Survival Is Shorter at Higher Temperature

Temperature has considerable impact on survival of submerged rice. In the one experiment where only temperature was varied, there was still 50 % survival at 20 °C after 15 and 28 days for IR42 and FR13A, respectively, whereas at 28 °C these durations decreased to 5 and 18 days (Adkins et al. 1990, glasshouse

experiments). Importantly, the ranking in submergence tolerance of the two varieties was not affected by temperature. Field experiments indicate similar trends in comparisons between seasons (Das et al. 2009), but remain equivocal since several other factors would have varied between seasons. More definitive answers in the field may be achieved by varying temperature only, using isolation of water columns in transparent chambers, as was successfully done when separating effects of micro-organisms and rice on O_2 concentrations in deepwater fields in Thailand (Setter et al. 1988b).

6.3 Responses After Desubmergence

6.3.1 Water Deficits

Symptoms of water deficits, such as rolling and wilting of leaves, were first observed in submergence-intolerant genotypes at 1.0–1.5 h after desubmergence, when the water level in tanks had been lowered to 10–20 cm, and these leaves then desiccated (as observed by Ismail and Setter at IRRI and Greenway at Ayuttaya, pers. comm. and pers. obs.). Genotypes classified as submergence tolerant did not show these symptoms, or much less so. This section first discusses the molecular biological evidence for the importance of water deficits, then the only measurements of water relations available for the leaf developed during complete submergence of rice are summarised, and suggestions are given for further physiological research on water relations of shoots and roots upon desubmergence.

Following desubmergence, as well as with non-submerged plants when the water potentials of the medium were decreased, three transcripts usually associated with drought were increased twofold in the six lines with *SUB1A-1* compared with the wild type (Fukao et al. 2011). Importantly, lines containing *SUB1A-1* were not only more tolerant to complete submergence but also more tolerant to drought imposed by drying soil, or by exposure of roots to high external osmotic pressure derived from polyethylene glycol (Fukao et al. 2011).

Severe water deficits were confirmed in glasshouse experiments following desubmergence after submergence for 4–6 days, using intolerant IR42 (Setter et al. 2010). The leaf which had developed during submergence desiccated within 60 min of desubmergence, while the water potential of the leaves dropped to below −1.5 MPa despite tight stomatal closure (Setter et al. 2010). The main cause of the water deficits in this youngest leaf blade that expanded during submergence was attributed to the development of very high resistances to xylem flow in the sheath, as indicated by the large water potential gradient between the blade of this leaf and the rest of the shoots (Setter et al. 2010). Leaf desiccation was prevented when plants were desubmerged at 100 % relative humidity. Nevertheless, there was no repair of the apparent lesion in the xylem as shown by transfer from 100 % to 50 % relative humidity at 5 days after desubmergence; within 15 min the leaf which had expanded during submergence dehydrated and its water potential dropped from −0.75 to −2.5 MPa (Setter et al.

2010). In contrast, leaves produced after desubmergence had similar water potentials to the leaves of non-submerged plants (Setter et al. 2010). Water deficits after desubmergence have so far not been tested in other studies.

Future studies might be aimed at further elucidation of the water deficits shown to occur upon desubmergence in leaves that had elongated when under water. Another main theme might be possible increases in hydraulic resistance in the roots. The only indication so far for such an increased resistance are the mild decreases in water potential from −0.4 to −0.65 MPa of leaf 2, which had been formed before submergence and was therefore unlikely to develop increased xylem resistance in the sheath, as observed in leaf 3 which expanded during submergence (Setter et al. 2010). Recently, a review concluded that decreases in root hydraulic conductivity often occurred during waterlogging and hypoxia (Aroca et al. 2012), although another review (Colmer and Greenway 2011) concluded that decreases in hydraulic conductivity, frequently reported for roots under waterlogging or hypoxia, were either transient over the first few hours of exposure, or due to long-term adverse effects such as cell death and plugging of xylem vessels (Bramley and Tyerman 2010). Nevertheless, adverse effects in the roots are possible since these might suffer anoxia when the shoots are submerged, particularly at night or in areas with turbid floodwaters (e.g. Winkel et al. 2013). Adverse effects of anoxia might be particularly acute when tissues have become low in carbohydrates (Gibbs and Greenway 2003), since as mentioned before carbohydrate starvation is likely in intolerant genotypes. Interestingly, submergence-tolerant lines, i.e. containing *SUB1A-1*, maintained better root growth during complete submergence (A.M. Ismail, unpublished).

Summing up, the key question for the period after desubmergence remains the extent to which poor growth, or even collapse of the plants, is due to carbohydrate exhaustion and/or chlorosis during submergence, and/or death of the foliage due to desiccation upon desubmergence. Of course, all may occur in concert, and so their relative contributions to the injury should be determined. Desiccation of leaves of plants already depleted of carbohydrates is also likely to slow, or prevent, recovery from carbohydrate starvation following desubmergence; as well low carbohydrates are likely to delay vigorous regrowth of plants with a severely reduced leaf area. Lodging after desubmergence may be another possible contributing factor to intolerance of varieties that elongate (Jackson and Ram 2003), although the evidence remains rather anecdotal and any lodging might be associated with loss of turgor due to water deficits (Setter et al. 2010), as well as to structural changes that weaken the cell walls in the elongated sheaths.

6.3.2 Free Radicals of Oxygen

There are numerous papers presenting evidence that oxidative injury following desubmergence can be an important component of the syndrome of intolerance to submergence (Jackson and Ram 2003). Before discussing possible injury due to ROS during, or after, submergence, it is opportune to emphasise that ROS

formation and hence any oxidative injury can have a large range of causes (Smirnoff 1995). For example during and/or after complete submergence of rice, formation of ROS could be triggered by previous very low to zero O_2 concentration followed by O_2 entry upon desubmergence, but also by dehydration of leaf tissues following desubmergence. So, any damage from ROS is almost certainly a consequence of more primary causes of cellular dysfunction discussed in this review. All the same, removal of ROS might prevent further damage by providing a suitable period of repair, as shown by incisive experiments on animal cells, which demonstrated that ROS can aggravate injury due to other causes, so that antioxidants can assist in recovery (Arthur et al. 2008).

Evidence for the importance of damage by ROS to submergence tolerance is usually based on comparisons between tolerant and intolerant genotypes. Physiological evidence consists of 1.7- to 3-fold more MDA, a lipid peroxidase product, in the intolerant than in the tolerant lines (Ella et al. 2003b; Fukao et al. 2011; Xiong et al. 2012). Also, during complete submergence ethane, another product of lipid peroxidation, was twofold higher in the intolerant than in the tolerant genotype, both during submergence and exposure to 0.2–0.75 % O_2 in a gas phase (Santosa et al. 2007). Consistently, H_2O_2 was 1.7-fold higher in the intolerant than tolerant line (Xiong et al. 2012), while Fukao et al. (2011) detected more H_2O_2 and O^{2-} in the intolerant than in the tolerant genotype (qualitative tests only). Ella et al. (2003b) could not detect a difference in H_2O_2 between genotypes; nevertheless, a better antioxidant system in tolerant FR13A was indicated since this genotype contained 2.5- to 3-fold higher activity of glutathione reductase, an enzyme of the ascorbate reduction cycle, than the intolerant genotype. Further, desubmerged FR13A, though having reductions in concentrations of reduced ascorbate, retained 2.5- to 3-fold higher concentrations of reduced ascorbate than IR42; reduced ascorbate is a well-known scavenger of free radicals of O_2 (Ella et al. 2003b). The lower levels of ROS (and/or associated damage) in submergence-tolerant genotypes are likely to be due to a combination of less ROS production and better scavenging systems.

An additional reaction, in addition to the well-known antioxidant systems to scavenge ROS (e.g. summarised in Smirnoff 1995), is that catalysed by catalase: ethanol + $H_2O_2 \rightarrow$ acetaldehyde + $2H_2O$ (reviewed by Boamfa et al. 2005). Experimental evidence for these reactions in vivo is the higher acetaldehyde formation in the tolerant FR13A than in an intolerant genotype, during submergence and when exposed to 0.05–0.2 kPa O_2 in a gas phase (Boamfa et al. 2005). The alternative source of acetaldehyde, pyruvate, is unlikely, since transfer to anoxia decreased acetaldehyde formation while ethanol formation became similar in the two genotypes (Boamfa et al. 2005). Acetaldehyde bursts also occur when plants first exposed to anoxia or micro-concentrations of O_2 are transferred to aerated conditions (Boamfa et al. 2005).

Further evidence for the amelioration by scavenging of ROS after desubmergence was obtained by exposing seedlings to 10 mM Na-ascorbate at pH 10 during the last day of 8 days of submergence; then survival increased from about 70 to 100 % in FR13A and from 40 to 100 % in the intolerant genotype IR97

(Thongbai and Goodman 2000). The higher level of ERP signals (electroparamagnetic resonance, which give indications of free radical activity) in the submergence-intolerant genotype was unfortunately only measured at 10 days after desubmergence (Thongbai and Goodman 2000), so is little more than an indication of irreparable injury.

The suggestions from the physiological observations that ROS are an important component of intolerance to submergence are strongly supported by molecular evidence. Firstly, when treated with viologen, which stimulates production of ROS in chloroplasts, the line with *SUB1A-1* had approximately double the increases in transcripts of ascorbate peroxidase, superoxide dismutase 1A, and catalase A, relative to the wild type (Fukao et al. 2011); all these enzymes are involved in removing ROS. So, these authors concluded that *SUB1A-1* augmented the antioxidant systems. Nevertheless, that cannot be the complete narrative, as shown by poor survival after desubmergence and larger decreases in chlorophyll during 13 days submergence in Goodah Heenati compared with FR13A, even though both contained *SUB1A-1* (Xiong et al. 2012). Molecular analysis showed these two genotypes had the same expression of transcripts for GA responsiveness and anaerobic metabolism (Xiong et al. 2012).

The damage after return to air is possibly associated with damage to mitochondria leading to leakage of electrons when O_2 again becomes available (Smirnoff 1995). An energy crisis can also lead to loss of membrane integrity during anoxia, as shown for anoxic potato tissue, attributed to action of lipolytic acyl hydrolase (Rawyler et al. 1999, 2002). This suggestion was based on free fatty acid release of two fatty acid chains per phospholipid hydrolysed and the absence of lysophospholipids, the products of phospholipids (Rawyler et al. 1999). We suggest such damage might also occur during the severe energy crisis at micro-concentrations of O_2, be it in the whole plant (Boamfa et al. 2005) or in localised regions. When some O_2 is available, the damage to the mitochondria might be amplified by the production of ROS. This hypothesis implies that exposure to micro-concentrations of O_2 would be more injurious than exposure to anoxia.

In conclusion, the reviewed data emphasise the adaptive/acclimative importance of systems scavenging ROS, for both re-aeration after exposure to anoxia and during occurrence of micro-concentrations of O_2, likely to occur at least in some tissues of submerged plants. Interpretations in future work might be aided from expression of parameters on a more suitable basis than fresh weight, which remains equivocal; especially in view of water deficits after desubmergence (Setter et al. 2010) and also since differences in leaf elongation may result in varying % of cytoplasm of the cell volumes. Enzyme activities and even levels of reserve carbohydrates may be expressed on a protein basis, which seems to be the simplest way to assess the metabolic machinery of plants differing in development, e.g. treatments or varieties that differ in rate of leaf elongation or hydration. Persuasive evidence for this notion has been shown for anoxic maize roots, up to 3.5-fold differences in maximum catalytic activity of ADH on a fresh weight basis between 5 mm root tips and the rest of the root axes were only 1.15-fold when expressed on a protein basis (Andrews et al. 1994).

6.4 Summary

Integration of results on physiological experiments with genetic and agronomic experiments is often hindered by the very different methods used in these disciplines.

Complete submergence of rice crops might be even more catastrophic than during the controlled field pond experiments described here and even more so than in controlled-environment experiments. In the field, complete submergence usually lasts from a few days up to 3 weeks, and there are sometimes two or more floods during the one season (Mackill et al. 2012). Furthermore, large variations in the characteristics of the floodwater such as CO_2, O_2, turbidity and irradiance (Sect. 2) will determine the % contribution to the overall effects by carbohydrate exhaustion and anaerobiosis, and so will depend on the location and flooding event. As depicted in Fig. 5 and its caption the most important variables may be irradiance, CO_2 levels and turbulence in the floodwaters.

Floods result in complex submergence environments, but this highlights the elegance of the *SUB1A-1* allele in adapting rice to this range of conditions, via its regulation of a number of genes contributing to tolerance. *SUB1A-1* suppresses ethylene signalling and diminishes shoot elongation. For the elongating genotypes, severe carbohydrate depletion has been implicated as the cause of death, but several additional factors might act in concert with this exhaustion of substrates, with their relative importance depending on the floodwater environment of particular locations. The additional factors include adverse effects from O_2 deprivation during submergence and water deficits and damage from ROS upon desubmergence, with the combination of factors presumably resulting in tissue injury.

Future experiments may benefit from more detailed time sequences and physiological measurements after desubmergence, rather than the customary survival ratings several days after desubmergence, ranging in different experiments between 6 and 21 days after desubmergence (Toojinda et al. 2003; Fukao et al. 2006; Xu et al. 2006; Ella et al. 2003a). These ratings are sometimes merely based on 'the development of at least one leaf'. Both the timing and criterion used are suited to large-scale breeding programmes; however, investigations focused on processes leading to survival, or death, need more and earlier observations after desubmergence (which preferably would be supplemented with manipulative treatments, such as supply of carbohydrates and mineral nutrients).

7 Conclusions and Perspectives

7.1 Dynamic Interchange Between the Disciplines of Genetics and Physiology

The present review, with its focus on physiology, complements the literature on the genetics of submergence tolerance, and molecular changes in gene expression

(cited in Introduction). The present review provides some examples of how the integration of physiology and genetics can lead to a clearer understanding of the adaptation–acclimation to flooding. An additional good example of an integrated approach is a microarray study by Lasanthi et al. (2007) of aerated and anoxic rice coleoptiles: 1,346 transcripts were upregulated and 1,770 downregulated, but these authors interpreted several of these changes in terms of their extensive knowledge of metabolism under anoxia; otherwise the data would have remained as merely a catalogue. Other examples of the complexity of the genetic changes and the potential usefulness of the integration between genetics and physiology come from QTL studies (e.g. Toojinda et al. 2003). So, the most efficient way forward may well depend on a dynamic interchange between physiological and genetic (including molecular) studies.

7.2 Physiological Mechanisms of Flooding Tolerance

This review has evaluated the current knowledge of physiological mechanisms in rice for tolerance to floods deeper than 0.3 m. Such floods impose penalties on growth and yields of rice in comparison with the shallower 0.05–0.2 m standing water in irrigated rice paddy considered in our companion review (Kirk et al. 2014). Diametrically opposing adaptations are needed to survive transient (days to weeks) complete submergence versus sustained deep floods during which some foliage is emergent (i.e. during partial submergence).

During long-term flooding internode elongation to restore and maintain emergent foliage and development of a gas-filled pathway through which O_2 and CO_2 can diffuse is essential for survival. In the case of internodes, this pathway delivers gases beneath floodwaters through what might be termed a 'snorkel'. Stem extension is promoted by ethylene signalling gibberellic acid-induced internode growth, involving intercalary meristems and enhanced elongation of the cells produced, supported by accelerated carbon flow to the internodes, both from starch hydrolysis and photosynthesis. Less is known to what extent variations in gas flow to underwater plant portions affect carbohydrate delivery to grain on the panicle (above the water), although severe limitations are indicated by low harvest indices of deepwater rice. Long-term studies are called for on carbohydrate metabolism in deepwater rice, exploring the possibility of photosynthesis in underwater portions and of enhanced rates in the emergent foliage, while the photosynthate supply available to roots is also a knowledge gap. Indeed, much less is known about the repercussions of deep floods on root function and survival, as compared with data available for shoots. Anecdotal evidence suggests that the sediment root system often decays, possibly due to anoxia. Equally importantly, we have very little understanding of the function of the profuse aquatic roots, which in some cases replace decaying sediment roots.

In contrast with those rice ecotypes with internode elongation as a key adaption to grow and yield in long-term floods, shoot elongation during transient floods

lasting a few days to 3 weeks leads to a catastrophe. Conclusive proof for this notion is provided by the great adaptive advantage endowed by the *SUB1A-1* allele, a mutation of the *SUB1A* gene, which encodes for an ERF transcriptional regulator. The mutation causing ethylene insensitivity and thus suppressing elongation by sheaths and leaves, dramatically enhances survival compared to elongating genotypes. For the elongating genotypes, severe carbohydrate depletion has been implicated as the cause of death, but this might not always be the case. We suggest several factors might act in concert, their relative importance depending on the environment in the floodwater of the particular fields. Such contributing causes may include adverse effects from O_2 deprivation during submergence and upon desubmergence water deficits and damage from free radicals, both resulting in aggravation of the injuries caused by the combination of factors. Supply of exogenous substrates, although technically challenging, would be a good test whether carbohydrate starvation can be prevented.

The large range of factors influencing tolerance to complete submergence and the substantial variations in responses based on characteristics of floodwater in farmers' fields also highlight that *SUB1A* might have to be supplemented by other genes. That main suggestion is based on the already mentioned 35–70 % of variation in survival, which could be attributed to *SUB1A-1* (Sect. 6.2). A likely possibility is that though *SUB1A* stimulates genes involved in coping with the various aspects of the complete submergence syndrome, the target genes might differ in degree of responsiveness and hence the variability not accounted for by *SUB1A* alone. Moreover, other genes are likely to add to the effect of *SUB1A-1* resulting in tolerance of longer flood duration, as suggested by the additional minor QTLs identified from FR13A and other donors (Nandi et al. 1997; Septiningsih et al. 2012).

The role of underwater photosynthesis in submergence tolerance has received relatively little attention. Gas films lining submerged leaves enhance photosynthesis, resulting in higher internal O_2 concentrations during the day, and the gas films also facilitate O_2 entry from floodwaters during nights. These beneficial effects are attributed to less resistance to O_2 and CO_2 fluxes between bulk solution and leaves. The possibility of anaerobiosis, at least in roots, during submergence also requires further tests; dovetailing investigations using mutants deficient in anaerobic catabolism with physiological–biochemical studies might provide the best way forward. Also emphasised is the key importance of using the appropriate basis of expression, particularly when comparing genotypes or plants from different treatments; e.g. a protein basis may be far more revealing than the more customary fresh and dry weight bases. The exploration of possible water deficits after desubmergence is still in its infancy and merits further study. For future physiological experiments, we have drawn attention to the imperative to evaluate events very shortly after desubmergence, i.e. within minutes or hours after desubmergence, as well as after several days and weeks of recovery.

7.3 Synopsis

In view of the vast range of conditions in various flooded environments, it is opportune to reiterate some highlights resulting from the current review:

1. Underwater leaf gas exchange has only recently been explored in some depth, but is crucial for photosynthesis and therefore also the internal O_2 provision of submerged plants, particularly to the roots.
2. Very unexplored aspects are the efficiency and survival of the sediment roots during flooding under different conditions such as irradiance and turbulence, and to what extent the aquatic roots can replace the likely damaged sediment roots.
3. Understanding of the shoot elongation process and implications for submergence tolerance is well advanced; in contrast the understanding of likely associated changes in carbohydrate status requires further elucidation and is crucial for understanding the mechanistic basis of survival, recovery, and yields. Little is known about rates of photosynthesis under field conditions, possible importance of sediment-derived CO_2, and the dose–response curve between irradiance and survival.
4. We have demonstrated that the disastrous consequences of transient complete submergence, though to a large extent alleviated by the *SUB1* QTL, are nevertheless complex. These complexities include possible carbohydrate starvation and involvement of anaerobic catabolism. A large deficiency in our knowledge is to what extent the catastrophe can be prevented, or delayed, by even very moderate irradiance and whether in waters of low CO_2 concentration, the fate of the plants might depend on at least some CO_2 supply from the sediments.
5. Detailed knowledge on the recovery after desubmergence appears to be lacking. Here, adverse effects of poor water relations of new leaves and the condition of the roots as impacted during submergence may both be of importance for shoot growth during recovery. In addition, scavenging systems of ROS may also greatly assist in recovery.

Overall, the various facets enumerated above might well differ in intensity depending on environmental conditions in the floodwaters of various locations and flooding events.

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Appendix: Does Appreciable O_2 Deficiency Occur in Submerged Rice in Flooded Fields?

Jackson and Ram (2003) concluded that anoxia is of minor importance during complete submergence of rice, while we suggest that its contribution to the syndrome, though still far from clear, may be quite substantial. The extent and thus possible importance of anoxia would depend on environmental factors which will affect photosynthesis and hence O_2 provision, being irradiance, CO_2 concentrations, and turbulence. Hence, a detailed consideration of some of Jackson and Ram's arguments is appropriate. This discussion also will highlight some of the difficulties in interpretation of experiments on this complex phenomenon.

The first argument was that when rice plants in soil were placed in the dark, they survived submergence at 0.05 mM O_2 for 7 days (Ellis and Setter 1999, criterion: some growth at 7 days after desubmergence), an O_2 concentration considered lower than usually encountered in the field (Jackson and Ram 2003). However, a study of the floodwaters in Indian fields showed that two out of six profiles had 0.092 mM or less O_2 at the water surface and 0–0.02 mM at the soil surface (Ram et al. 1999), indicating the likelihood of much more serious deficiencies of O_2 in the field than in the hypoxic solutions of Ellis and Setter (1999), particularly since Ellis and Setter used gas flushed solutions, rather than the usually less turbulent solutions encountered in the field.

The second set of experiments to be evaluated was with rice seedlings (Boamfa et al. 2005). There were some anoxic exposures, but also submergence in water with some O_2 available. Absence or presence of ethanol and acetaldehyde emissions was taken to indicate whether there had been anoxia during submergence. However this criterion is far from ideal. Firstly, if there is little emission of these products of anaerobic catabolism, during submergence under O_2 deficits rather than anoxia, the rate of evolution of formed ethanol and acetaldehyde may be severely underestimated when parts of the tissues receive sufficient O_2 for oxidative phosphorylation and therefore could consume the acetaldehyde and ethanol produced by the anoxic regions. Secondly, evolutions of acetaldehyde and ethanol after desubmergence, which were substantial over 6 h re-aeration after treatments longer than 18 h, may not be indicative of the rate of anaerobic catabolism during anoxia, but rather to inhibition of mitochondria due to injury incurred during the submergence, or desubmergence (cf. Gibbs and Greenway 2003).

The third line of evidence quoted by Jackson and Ram (2003) comes from an experiment with shoots of submerged rice, which were at high irradiance during the 12 h light cycle (Waters et al. 1989). In that experiment, cessation of root elongation during the night was rapidly reversed as soon as light was again available (Waters et al. 1989). However, these experiments were in clear water with high irradiance not only through the water surface but also from the sides of a 70-cm-long transparent cylinder surrounding the leaves, while nutrient agar around the roots did not provide the usual sink for O_2 encountered in soils.

In conclusion, severe O_2 deficits cannot be excluded particularly in the root tips of submerged rice in the field (see also Winkel et al. 2013).

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Part IV
Ecology

Beyond Mutualism: Complex Mycorrhizal Interactions

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Abstract The soil–plant–fungal matrix is inherently complex. There are thousands of species, highly variable environments in time and space, multiple interactions within a range of resources, and exchanges between multiple trophic levels. Here, we look at the structure of that complexity to see if there are emergent properties that allow us to understand that complexity. We emphasize our work from Mediterranean-type ecosystems in California and Oregon, but the perspective is valid across biomes. We examine a diversity of mycorrhizal types, with an emphasis on interactions of arbuscular mycorrhizae (AM) and ectomycorrhizae (EM) with host plants. These taxonomically different fungi differ in structural and biochemical properties including hyphal growth patterns and enzymatic capabilities. Because the symbionts are fungi, their hyphae connect multiple plants, forming networks. Materials (C, N, P, and water) are exchanged between plants through mycorrhizal networks. Importantly, networks themselves have structural properties that confer stability or instability and control the directions of flows. Thus, network theory has the ability to resolve patterns of elemental transfers and exchanges, and thus the outcomes for plant community dynamics. Also importantly, hyphae and fine roots have limited life spans, making interactions highly dynamic. Together, these dynamic interactions will help us unravel the complex relationships and the evolutionary histories that result in community and ecosystem dynamics.

1 Introduction

The soil–plant–fungal matrix is inherently complex. In plant–fungal interactions, complex systems include more players, more interactions, and emergent properties (Allen et al. 2003, Southworth et al. 2005; Southworth 2012). Complexity refers to nonlinear relationships, networks, feedback loops, multiple interactions at single and multiple trophic levels, and functional uncertainty or variability.

In mycorrhizal symbioses, plant roots and fungi interact in close physical contact to form three-dimensional structures with varying degrees of temporal and spatial stability. The relationship is generally considered mutualistic with plants providing carbon to fungi and mycorrhizae providing mineral nutrients and water to plants (Smith and Read 2008).

Mycorrhizae are classified into two broad groups: ectomycorrhizae (EMs) and arbuscular mycorrhizae (AMs). Ectomycorrhizae form with fungi in the Basidiomycota, Ascomycota, and Zygomycota, and with hosts predominantly in the Pinaceae, Fagaceae, Betulaceae, Myrtaceae, and woody Rosaceae. Structurally, EMs develop a fungal mantle, coating the root tips; hyphae penetrate between cells of the surface layers of the root, surrounding root epidermal cells with a Hartig net. Arbuscular mycorrhizae form with fungi in the Glomeromycota; most herbaceous plants form AMs as do many woody species. In AMs, hyphae penetrate the root cortex and form arbuscules (ramified structures) within root cortical cells and

vesicles between root cells. In both AMs and EMs, hyphae extend outward into soil interstices where they interact with organic and inorganic nutrients and with other soil microorganisms. Some EM hosts also form AMs. Ericoid and arbutoid mycorrhizas are subclasses of EMs (Berch et al. 2002; Smith and Read 2008).

Here we focus on the complex interactions of mycorrhizal networks predominantly in the Mediterranean-climate ecosystems of California and southern Oregon where climate imposes strong seasonal differences in temperature and moisture with hot dry summers and cold wet winters. This Mediterranean-climatic zone offers ideal ecosystems in which to study complex mycorrhizal–plant–soil interactions.

These ecosystems consist of oak (*Quercus* spp.) woodlands with one or two dominant species of widely spaced perennial EM oaks and pines in a matrix of annual AM grasses and native forbs. They are "simple" in the sense that they contain relatively few EM species. This apparent simplicity aboveground is desirable for studying biocomplexity belowground. The belowground structure of oak/grass woodlands includes mycorrhizal root systems that form interacting networks, with diverse functions, heterogeneous soil nutrients changing over time, and plant systems whose carbon supports the complex belowground architecture.

Based on our work, several conceptual advances alter the way we view mycorrhizal interactions. (1) In semiarid ecosystems, EM diversity includes more fungal species with resupinate and hypogeous sporocarps. (2) Hyphal diversity differs from both EM root tip and sporocarp diversity. (3) General network theory can be applied to mycorrhizal networks. (4) Water taken up by hydraulic lift can be transferred via mycorrhizal networks. (5) Hyphal networks transfer nutrients among plants, but proximity of plants to one another matters little. (6) Ectomycorrhizal fungal species differ in the relative amounts of C and N exchanged and may not benefit their hosts equally. (7) Mycorrhizal associations are dynamic, forming, breaking down, and reforming over diverse life spans.

2 Diversity of Mycorrhizal Fungi in Western North America

Mycorrhizal fungal communities with *Quercus* species in the Mediterranean ecosystems of western North America are dominated by hypogeous Ascomycota and epigeous Basidiomycota, as determined by sporocarp collections and by DNA sequencing of mycorrhizae (Table 1). In contrast to temperate ecosystems, sporocarps collected in northern CA *Q. douglasii* woodlands had a higher proportion of resupinate taxa such as *Tomentella* spp. and Sebacinales (Smith et al., 2007). The relative frequency of sporocarp types was 23 % resupinate, 24 % hypogeous, 31 % epigeous, and 22 % unknown or asexual (Smith et al. 2007). Although other studies reported a disconnect between fungi as EMs and as sporocarps (Gardes and Bruns 1996; Horton and Bruns 2001), this was not the case in the *Q. douglasii*

Table 1 Mycorrhizal fungi associated with *Quercus* species in Mediterannean ecosystems of North America identified by DNA sequencing (1, present on that species): *Q. crassifolia* (Morris et al. 2008a, 2009), *Q. laurina* (Morris et al. 2009), *Q. douglasii* (Smith et al. 2007; Morris et al. 2008b), *Q. wislizeni* (Morris et al. 2008b), and *Q. garryana* (Valentine et al. 2004; Frank et al. 2009; Moser et al. 2009)

Quercus species							
Fungal taxon	Fb	*crassifolia*	*laurina*	*douglasii*	*wislizeni*	*garryana*	Frequency
Ascomycota							
Cenococcum	h	1	1	1	1	1	5
Genea	h	1	1	1	1	1	5
Tuber	h	1	1	1	1	1	5
Helvella	e	1		1	1		3
Pachyphloeus	h	1	1	1			3
Peziza infossa	h			1	1	1	3
Tarzetta	h	1		1		1	3
Genabea	h			1	1		2
Otidia	e			1		1	2
Balsamia	h					1	1
Cazia	h					1	1
Geopora	h					1	1
Gilkeya	h					1	1
Hydnoplicata	h				1		1
Marcellinia	h			1			1
Trichophaea	e					1	1
Basidiomycota							
Cortinarius	e	1	1	1	1	1	5
Inocybe	e	1	1	1	1	1	5
Lactarius	e	1	1	1	1	1	5
Russula	e	1	1	1	1	1	5
Sebacinales	r	1	1	1	1	1	5
Thelephoraceae	r	1	1	1	1	1	5
Clavulinaceae	e	1	1	1	1		4
Hebeloma	e		1	1	1	1	4
Xerocomus	e	1	1		1	1	4
Amanita	e	1	1		1		3
Hygrophorus	e	1			1	1	3
Boletellus	e	1	1				2
Cantharellaceae	e	1	1				2
Entoloma	e	1		1			2
Gymnomyces	h			1	1		2
Hydnobolites	h			1	1		2
Laccaria	e			1	1		2
Scleroderma	h		1			1	2
Tricholoma	e	1	1				2
Antrodiella	r					1	1
Astraeus	e					1	1
Atheliaceae	r	1					1
Boletus	e					1	1

(continued)

Table 1 (continued)

Quercus species							
Fungal taxon	Fb	*crassifolia*	*laurina*	*douglasii*	*wislizeni*	*garryana*	Frequency
Clavariaceae	e				1		1
Coltricia	r	1					1
Gautieria	h			1			1
Hydnum	e	1					1
Hysterangium	h			1			1
Melanogaster	h				1		1
Octaviania	h			1			1
Ramaria	e		1				1
Sistotrema	r	1					1
Total taxa		24	19	25	23	24	

Fruiting body (fb), morphology is epigeous (e), hypogeous (h), or resupinate(r)

woodland where there was a 45 % match (Smith et al. 2007). In semiarid woodlands with Mediterranean climates, it is necessary to sample inconspicuous sporocarps including hypogeous and resupinate types.

2.1 Diversity of Mycorrhizal Hyphae

Few studies have compared the diversity of the EM hyphal community with that of sporocarps or EMs (but see Smith et al. 2007). Hyphal sampling methods determine which fungal species are detected (Suz et al. 2008). For example, with the in-growth bag method, certain species, e.g., *Cenoccocum geophilum* and *Tuber* spp., are not found; both are contact hyphal exploration types that do not grow extensively into soil or in-growth bags (Hynes et al. 2010).

We compared hyphal communities beneath *Quercus douglasii and Q. wislizeni* with those of *Pinus sabiniana*. Hyphal species were matched to those found as sporocarps and as EMs at the same site (Hynes et al. 2010; Smith et al. 2007). Hyphae in root-restrictive in-growth bags in soil were identified. Among the 33 species detected, EM rhizomorph-forming fungi (e.g., Thelephoraceae and Boletales) dominated the hyphal communities (Hynes et al. 2010). Most fungal taxa in soils near *Quercus* spp. and *P. sabiniana* were Basidiomycota, while taxa near *P. ponderosa* included more EM Ascomycota and non-mycorrhizal fungi. There was little overlap in fungal species composition among the trees species. Composition of the EM hyphal community was related to host species, based on canonical correspondence analysis. Many EM species present as hyphae also were collected from this site as sporocarps (18 %) or as mycorrhizae (58 %) (Smith et al. 2007; Morris et al. 2008a). However, the hyphal community was dominated by taxa that differed from the sporocarp and mycorrhizal communities (Hynes et al. 2010).

Similarly, in an old-growth *Quercus ilex* forest from Corsica, EM diversity was large (140 taxa) and dominated by *Cenococcum geophilum* (35 %) with many rare

species (Richard et al. 2005). There was little overlap between above- and belowground EM species (<20 % overlap). Ericoid and arbutoid mycorrhizae are prominent in Mediterranean ecosystems. For example, in Italy, *Q. ilex* ectomycorrhizas harbored fungi that formed ericoid mycorrhizas on nearby ericaceous plants (Bergero et al. 2000). In Corsica, *Q. ilex* and ericaceous plants shared numerous mycorrhizal species and facilitated colonization and seedling survival (Richard et al. 2005, 2009). In southern Oregon, USA, *Arctostaphylos* species shared EM fungi with *Q. garryana* and with *Cercocarpus ledifolius* (Rosaceae) (McDonald et al. 2010).

3 Networking and Interplant Interactions

3.1 Mycorrhizal Networks

Mycorrhizal hyphae can extend from one plant to another, effectively linking plants belowground. This has led to the concept of a "common mycorrhizal network" where "common" refers to mycorrhizal fungal species held *in common* by two hosts and with the implication such linkages are "common" or frequent, not rare (Smith and Read 2008). The expression "mycorrhizal network" suffices to describe the belowground system of the interconnected hyphae of one or more fungal species and the roots of one or more plants of the same or different species. Mycorrhizal networks differ from "mycelial networks," an expression that refers to extraradical mycelium without regard for its mycorrhizal nature (Leake et al. 2005).

Mycorrhizal networks cannot be observed directly in natural settings because of the fragility of hyphal connections. However, fragments of the network consisting of hyphae joining the roots of two plants can be observed through transparent plates (Read et al. 1985; Read 1992) and in high-resolution minirhizotrons (Allen et al. 2007). Transfer of radioactive substances among hosts of the same and different species of both AM and EM plants has provided evidence for the functioning of mycorrhizal networks and also enabled visualization of hyphal linkages by autoradiography (Francis and Read 1984; Read et al. 1985; Read 1992). Studies using stable isotopes show that C, N, and P move among mycorrhizal plants, potentially via mycorrhizal networks (for reviews: Read 1997; Simard et al. 2002; He et al. 2003, 2006; Selosse et al. 2006; Montesinos-Navarro et al. 2012; Simard et al. 2012).

Wherever the same fungal species occur on the same or different host species, there is the potential for a mycorrhizal network. While it is not surprising that individuals of a given host species share the same fungal species and even fungal genets, the finding that hosts in different plant families share fungal species is more remarkable and indicates some selection for host-generalist fungi. Host-specialist fungi can isolate the host from other species and keep nutrients for only that host. Host-generalist fungi serve the broader plant community, integrating species and

promoting interdependence. In California oak woodlands, *Q. douglasii* (a deciduous oak) and *Q. wislizeni* (an evergreen oak) shared 13 out of the 16 most common EM fungi (Morris et al. 2008b). Shared fungal species have been observed on such diverse pairs as *Betula papyrifera* (Betulaceae)–*Pseudotsuga menziesii* (Pinaceae), *Quercus garryana* (Fagaceae)–*Cercocarpus ledifolius* (Rosaceae), *Helianthemum bicknellii*–*Quercus* species, and *Quercus douglasii*–*Pinus sabiniana* (Pinaceae) (Simard et al. 1997; McDonald et al. 2010; Dickie et al. 2004).

3.2 Network Theory

General network theory can be applied to mycorrhizal networks (Simard and Durall 2004; Southworth et al. 2005, Chagnon et al. 2012; Öpik and Moora 2012; Simard 2012). A network is a system of nodes connected by links such as cities connected by roads or airports connected by air routes. Nodes are stable points or structures; links are the connections between them. The architecture of a network includes the number of links per node, the degree distribution or probability that a node has a certain number of links, the directionality of links, and the path length or number of links needed to travel between two nodes (Barabási and Oltvai 2004).

In mycorrhizal networks, either plants or fungi might be considered nodes (Southworth et al. 2005). In the phytocentric view, plants would be the nodes and fungi the links. Transfer of nutrients among mycorrhizal plants supports this interpretation. Other signaling substances may also move between plants via mycorrhizal networks (Song et al. 2010). Alternatively, in the mycocentric view, the fungi (mycelium + sporocarps) would be the nodes and plants the links. Water and C as well as linked nutrients move from host plants into fungi (e.g., Querejeta et al. 2003). We know of no experiments demonstrating transfer of material substances among mycorrhizal fungi. Finally, mycorrhizal networks could be "affiliation networks" in which both plants and fungi would be nodes that are linked at mycorrhizal root tips.

One application of network theory to mycorrhizal networks is the determination of hubs. If the number of links per node is random or normally distributed, then no single node is more important to the network than any other, and the mean number of links per node is close to the median number. Alternatively, if a few nodes have exponentially more links than most other nodes, the network is said to be "scale free," and the nodes with the greater number of networks are hubs.

Translating this to mycorrhizal networks, if the number of fungi per tree is random, then no single tree is more important to the network than any other tree. Likewise for fungi, if the number of trees per fungus (the frequency of a particular fungal species) is normally distributed, then no one fungal species dominates. In *Quercus garryana*, the number of fungal species (morphotypes) per tree was random implying that no single tree was a hub (Southworth et al. 2005). However, the distribution of plant links to fungi was nonrandom and thus scale free, suggesting that certain fungi, in this case *Cenococcum*, may act as hubs. In an

examination of the linkages of genets of the *Rhizopogon vinicolor—R. vesiculosus* complex to individual trees of Douglas-fir (*Pseudotsuga menziesii*), the number of fungal genets per tree was scale free, indicating that certain trees were hubs—highly interconnected to many other trees (Beiler et al. 2010). Similarly the frequency of fungal genets on trees was scale free, indicating that certain genets were hubs, playing a greater role in forest tree connections. Scale-free networks suggest that mycorrhizae create "hub trees" by providing these trees with more connections to other trees.

3.3 Role of Mycorrhizal Networks in Seedling Establishment

Most seeds germinate without regard for the availability of mycorrhizal fungi. However, seedlings of most plants, particularly EM species, require colonization by mycorrhizal fungi for growth and establishment in field settings. Seedlings with roots that extend into a mycorrhizal network have access to existing mycorrhizal fungi, and those fungi are receiving C from other hosts. Thus seedlings initially have the benefit of mycorrhizal services, but do not have to "pay" for them.

For example, in oak (*Quercus garryana*) woodlands, seeds that germinate on the ground overlying the root-hyphal zone encounter the mycorrhizal fungi of the parent tree while seeds germinating beyond the root-hyphal zone do not (Table 2, Frank et al. 2009). In planted seedlings of *Q. agrifolia*, under the tree canopy, seedlings picked up a diversity of EM fungi, whereas those planted into the neighboring grassland were dominated by AM fungi, most likely linked to annual grasses and AM shrubs (Lindahl 2002). This apparent advantage of host proximity has its limitations, particularly if the parent tree reduces carbon fixation by shading the seedlings or by competing for water and nutrients. Thus, around oaks, there is a narrow zone of seedling success where seedling roots access mycorrhizal fungi, but are not outcompeted by the parent tree. Seeds that germinate beyond the root-hyphal zone of mature trees become mycorrhizal with hypogeous fungi that were likely to have been dispersed by small mammals. These distant seedlings would not compete with the parent tree for resources, but also would not be networked to other trees. As root-hyphal zones extend and overlap, they could link to other saplings or trees. All seedlings that survived were mycorrhizal—either with fungi from the existing mycorrhizal network or with hypogeous fungi dispersed by small mammals (Frank et al. 2009).

In beach pine (*Pinus contorta* var. *contorta*) at the seacoast in Oregon, at the mature forest edge, trees formed EMs with 25 fungal species (Ashkannejhad and Horton 2006). In the deflation zone where winds remove the sand down to the water table, seedlings formed mycorrhizae with 21 fungal species, 11 of which also occurred in the forest. Isolated seedlings further out on the dunes formed mycorrhizae with ten species of fungi, seven of which were also found in the forest. As with *Q. garryana* and *P. contorta* var. *contorta*, seedlings of Douglas-fir (*Pseudotsuga menziesii*) that were close to trees, but beyond the canopy edge,

Table 2 Mycorrhizal fungi at distances from host trees of *Quercus garryana* in southern Oregon (1, presence)

	Soil cores	Seedlings	Seedlings >20 m
Fungal taxon	Under canopy	Under canopy	From canopy
Astraeus	1		
Cazia[a]		1	
Cenococcum	1	1	
Geopora[a]		1	1
Hebeloma	1		
Scleroderma	1	1	
Tarzetta	1	1	
Tomentella	1	1	
Tuber candidum[a]	1	1	1

From Frank et al. (2009)
[a]Hypogeous sporocarps

had the greatest survival as well as the greatest fungal diversity (Teste et al. 2009a, b). Thus seedlings close to the parent mycorrhizal network have the greatest likelihood of establishing mycorrhizal connections.

3.4 Role of Mycorrhizal Networks in Plant Community Interactions

Clearly the ubiquity of mycorrhizal networks among diverse plant species and diverse mycorrhizal types as well as fungal species means that mycorrhizal networks influence plant communities (van der Heijden et al. 1998). The question is whether mycorrhizal networks can control who enters, who succeeds, and who dies out in a plant community. To what extent do mycorrhizal fungi write assembly rules for plant communities?

Controlled experiments in glasshouses and plots with small numbers of species over a relatively short time (compared to ecological succession much less evolution) show that the fungal partners influence plant outcomes (e.g., Allen 1991). How can one get to the big picture, to the large-scale set of interactions and linkages within a plant community?

Natural experiments have demonstrated host–fungal relationships in recolonizing volcanic "deserts." The eruption of Mount St. Helens in Washington, USA, in 1980 inadvertently demonstrated the role of mycorrhizal networks in revegetation of entire plant communities (Allen et al. 1984, 2005). Whole swaths of aboveground vegetation—herbaceous plants, riparian vegetation, and coniferous forests—were destroyed, but belowground mycorrhizal networks, seed banks, and small mammals survived. The first plants to return were *Lupinus lepidus* which were both rhizobial and AM. Initially the AM community had one species, *Glomus macrocarpum*; eventually other AMs arrived. *Salix* and *Alnus*, both of which are

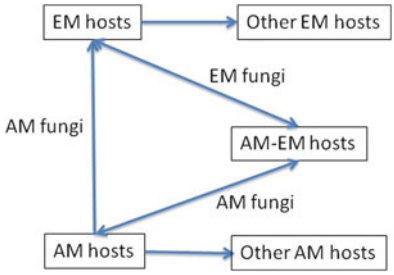

Fig. 1 Mycorrhizal network linkages among and within different host types (AM, EM, and AM-EM) and among host types

dual AM and EM, returned to streamsides, and finally the predominantly EM conifers (*Abies* spp.) germinated and reestablished forests. Arbuscular mycorrhizal herbaceous plants are not EM, though the roots may house endophytes (dark septate). *Salix* and *Alnus* are characterized by dual AM and EM colonization. *Abies* and other genera in the Pinaceae are extensively and predominantly EM, but include some AM hyphae and vesicles (see Smith et al. 1998) as well as dark septate endophytes. Thus with vegetation recovery occurring from seedlings inoculated by the existing mycorrhizal network, the entire vegetation of plant communities may be linked via a complex mycorrhizal network (Fig. 1). The first step in determining the potential for a community-wide mycorrhizal network would be to identify the fungal symbionts of (AM) herbaceous plants, of the dual AM-EM plants, and of the predominantly EM plants. Both molecular and microscopic approaches could be used, followed by experiments to demonstrate transfer of materials among the mycorrhizal types.

Similar patterns of revegetation with EM and AM species were observed on Mt. Fuji in Japan (Nara 2006a, b). *Salix* was the first colonizer on pumice deserts. Subsequently, *Betula* and *Larix* became established near *Salix* with some shared EM fungal species.

4 Water

Water is a critical resource in any terrestrial ecosystem. When in deficit, it is critical for plants to increase rates of water uptake. Other resources such as nitrate, calcium, or potassium, normally obtained through mass flow of water, can also limit plant production. Water throughput can be altered by physiological change in the host or by physical contact that is regulated by the architecture of the hyphal–root–soil interface. Furthermore, mycorrhizal fungi access water and transport water to plants, but they also utilize water obtained from plants if the conditions for reverse flow, a potential gradient, and barriers are not erected to restrict that flow (e.g., Allen 2008, 2009). The direction of flow goes beyond uptake for one plant and the hyphal matrix becomes of critical importance in plant–plant interactions.

4.1 Role of AM and EM Mycorrhizal Hyphae in Water Uptake

Stahl (1900) first postulated that EMs increase water uptake. Since the fungal mantle covers absorbing root tips, water (and nutrients moving via mass flow) must come through the fungus at greater rates than through uncolonized roots. Hatch (1937) demonstrated uptake of bound nutrients through hyphae, although labile nutrients continued to be taken up at greater rates than through hyphae. Increased water throughput correlates with both AMs and EMs (Allen et al. 1981; Allen 1982, 2009; Hobbie and Colpaert 2004). This increased water uptake altered competitive relationships in both glasshouse and field studies (Allen and Allen 1986).

Plants adapt to water stress by decreasing plant water potential and increasing stomatal conductance, in part through altering the hormonal activity of the host (Allen and Boosalis 1983). Different species of AM fungi differentially alter the water relations of a host, due to shifts in osmotic pressure (Augé 2001). Physiological changes could alter an individual plant making it more competitive with neighbors, but community-scale change would be expressed only through the impact of both symbionts (fungus and plant) in competition with their neighbors.

4.2 Direct Hyphal Transport of Water

Another way that mycorrhizae influence plant water relations is through the alteration of direct flow patterns. Direct hyphal transport of water from soil into plants increased water uptake and carbon gain in host plants (Duddridge et al. 1980; Allen 1982). Foliar $\delta^{18}O$ and $\delta^{13}C$ values of seedlings inoculated with native *Glomus* spores were best explained by an increased throughput of water in these plants (Querejeta et al. 2006, 2009). While mycorrhizae increase uptake of water, that water flow is not unidirectional. Water moves in response to osmotic gradients created by differential drying zones or differential solute concentrations (separated by membranes) and modulated by barriers such as the endodermis. Mycorrhizal fungi connect multiple plants with different architectures and change the structure of the soil with the potential to redirect water in complex, often unpredictable directions.

4.3 Hydraulic Lift

The complexity of mycorrhizal and community-scale interactions can be illustrated by the role of mycorrhizae in hydraulic redistribution of water (Allen et al. 2002). There are rapid diurnal fluctuations in soil water potential around oak roots in late summer. Soil water potential increases gradually at night and then rapidly decreases during daytime—a process known as hydraulic lift. In northern California, *Quercus douglasii* exhibited this phenomenon (Ishikawa and Bledsoe 2000). These fluctuations closely

followed diurnal fluctuations in soil temperature even at depths up to 100 cm. Hydraulic lift occurred too late in the summer to benefit annual grasses but could benefit oaks (Ishikawa and Bledsoe 2000). Water released by oak roots at night could slow the rate of soil water depletion, as well as allow nutrient uptake, increase oak nutrition, and extend their growing season.

4.4 Mycorrhizal Access to Water in Bedrock

In Mediterranean climates, groundwater or water in bedrock is critical to plant survival during the drought period (Allen 2011). As rainfall declines into the dry season, roots of some, but not all of the plants, track water depletion down the profile. Roots and mycorrhizal fungi extend even into granite and calcareous bedrock. In some cases, these roots are in bedrock, whereas in others, the roots search out perched water pockets (e.g., Hubbert et al. 2001; Querejeta et al. 2007b; Estrada-Medina et al. 2013). Mycorrhizal fungal hyphae track these roots through fractures in the rock and then extend from root tips penetrating the granite (Egerton-Warburton et al. 2003; Bornyasz et al. 2005). There is little nutrition in the granite matrix, so water appears to be the only available resource to the fungi (Allen 2006). Isotopic evidence indicates that plants utilize groundwater and water in bedrock well into the growing season (Allen 2006; Kitajima and Allen, unpublished data).

During the day, water is transpired. However, when surface soils are dry, too dry for growth of mycorrhizal fungal hyphae, water is redirected, by osmotic pressure gradients, from shoots to fine roots, and then into mycorrhizal fungal hyphae (Querejeta et al. 2003). Importantly, this water is redirected back to the plant when stomata open. But because hyphae connect multiple plants, water moves in the direction with the strongest gradient. In many cases, that direction is through hyphae into a neighboring plant sharing the mycorrhizal network with the deep-rooted host (Egerton-Warburton et al. 2007). In addition, some water is exuded from hyphal tips into bulk soil where it dissolves ammonium that is translocated throughout the mycorrhizal network (Allen 2006; Egerton-Warburton et al. 2008). In the field, that water sustains EMs throughout the growing season, whereas oak trees shift to AM associations under greater drought (Querejeta et al. 2007a). In topographic locations where trees access groundwater, a high diversity of EM fungi dominate the mycorrhizal community, supporting connected seedlings and increased productivity when surface soils are too dry to support that activity (Querejeta et al. 2009). On hillsides where roots cannot access deeper water, lower productivity exists; seedlings are rare; and AMs dominate.

Direct water movement through hyphae is a primary mechanism whereby the mycorrhizal network changes the growth patterns of multiple hosts. But another more subtle mechanism may be at work. Even dead hyphae preferentially conducted water compared with bulk soil (Querejeta et al. 2012). Both EM and AM fungi produce glomalin, chitin, and other compounds that enrich soil organic matter (Treseder and Allen 2000; Rillig and Mummey 2006). Decomposing hyphae

comprise a large fraction of the soil organic matter (Godbold et al. 2006). That organic matter would be left in place, connecting longer-lived roots with the previously connected mycorrhizal network. With fungicides, water transport still occurred (Querejeta et al. 2012); either dead mycorrhizal-network hyphae or residual organic matter from mycorrhizal-network hyphae regulated the pathway of water flow water retention. Thus, the role of mycorrhizal fungi in directing water and sequestering C appears critical. There still remain questions as to the quantitative roles of mycorrhizal networks in water and C transfers.

5 Nutrient Exchanges

Complex feedbacks that are regulated by availability and distribution of soil resources and by climate may operate among plant and fungal partners. Alteration of soil, plant, or fungal components may change structure and function of mycorrhizal networks. Changes in the three components may alter fungal and plant species composition and soil resource availability, resulting in long-term changes in plant community dynamics, soil stability, nutrient cycling rates, and ecosystem production.

Acquisition of nutrients depends not only on mycorrhizal hyphae and plant roots but also on water availability. Where water availability is seasonal, root activity is an additional consideration. Thus nutrient uptake and exchange among plants, fungi, and soils are complex interactions driven by fungal factors such as host specificity, fungal diversity and function, and fungal life spans—all moderated by water availability.

5.1 Nutrient Acquisition and Root Growth

Nutrient acquisition is strongly influenced by root growth, which differs seasonally for oaks and grasses in Mediterranean systems. Oak root and EM fungal growth occurs in spring and summer (even with summer drought), while grass root and AM fungal growth occurs primarily in fall and winter (Cheng and Bledsoe 2002). This spatial and temporal separation of nutrient acquisition partitions nutrients to different plant species with a lesser role for competition. Nutrients acquired by microbes (short-term) and by annual grasses (medium-term) eventually are acquired by perennial oaks (long-term) (Cheng and Bledsoe 2002). Thus in Mediterranean ecosystems with perennial species such as oaks and pines, nutrients become concentrated in the perennials and in soil under their canopies, while annual grasses and forbs become increasingly nutrient limited.

5.2 Nitrogen Acquisition and Mycorrhizal Diversity

Nitrogen and phosphorus are the two most common growth-limiting nutrients in many ecosystems. Factors affecting nitrogen acquisition are particularly complex since N exists in both organic and inorganic forms. Organic N forms are central to

N-cycling. Both AMs and EMs are efficient at organic N acquisition (Cheng and Bledsoe 2004, 2005; Hawkins et al. 2000; Jin et al. 2005).

Mycorrhizal diversity can affect N acquisition (see review by Leake et al. 2005). In mature *Q. douglasii* EMs, ^{15}N natural abundance differed by morphotype with $\delta^{15}N$ values from +0.2 to +4.3 suggesting that EMs accessed different soil N sources, i.e., inorganic N (less ^{15}N enriched) or organic N (more ^{15}N enriched). In a northern California field study with seedlings of three oak species (*Quercus douglasii*, *Q. garryana*, and *Q. agrifolia*), ^{15}N transfer from leaves to EMs differed by fungal species as judged by EM structure (morphotype), with greater acquisition by less common types (He et al. 2007). More abundant EMs included black (*Cenococcum* sp.), brown (Thelephoraceae), tan (Pezizales including *Tuber*, *Genea*, and *Helvella*), and white (*Hebeloma* and *Inocybe*) morphotypes. Of EMs, 75 % were colonized by black and brown morphotypes and were less enriched in ^{15}N than the other morphotypes (He et al. 2007).

The ability of AM plants to digest organic matter is unclear and studies are rare. Acquisition of N from complex organic matter was explored using novel experimental litter chambers ("litter wheels") (Stout 2004). Litter chambers contained one of two ^{15}N-labeled litter types from the annual grass *Bromus diandrus*, either root or shoot litter, and were covered with screens that allowed roots and hyphae (coarse screen) or hyphae alone (fine screen) to access the litter. Chambers were installed in the field adjacent to four plant types—annual grasses, forbs, legumes, and oak seedlings. Plants and chambers were harvested after 3 or 12 months. All plant types were more enriched when exposed to higher quality (greater % N) shoot litter than with root litter. Over time (short-term, 3 months; long-term, 12 months), forbs and grasses became more enriched in ^{15}N than did legumes or oaks (Stout 2004). The extensive fibrous root systems of grasses may have enabled greater access to ^{15}N-rich litter. Results of screen size (coarse or fine) were unexpected. Forbs and legumes acquired more ^{15}N from fine-screen than from coarse-screen litter wheels unlike grasses. Here forbs and legumes relied more on hyphal access to litter wheels, perhaps due to their less fibrous root systems. Oaks with thicker woody roots and predominantly EM colonization were poor competitors compared to grasses, forbs, and legumes.

5.3 Hyphal Mediation of Nutrient Acquisition

Do hyphae mediate transfer of nutrients between plants, and is transfer direct (within hyphae) or indirect (through soil)? In a field study, nutrients moved from *Quercus douglasii* seedlings to EM hyphae and from EM hyphae to receiver oaks (Meding 2007). Donor and receiver oaks were separated by buried paired 25-μm stainless steel mesh screens separated by a 1-cm air gap to prevent nutrient transfer via bulk flow in soil. Ectomycorrhizal colonization of oaks was high (94 %) with yellow and black morphotypes most common. Molecular analyses indicated the presence of *Cenococcum* spp. and *Tuber* spp. as well as AM fungi (Glomales;

Table 3 Transfer of ^{15}N and Rb (as analog for K) from leaves of donor oak seedlings to receiver oaks and grasses separated from donor by 25-μm mesh screens with a 1-cm air gap

	Donors		Receivers	
Plant part	$\delta^{15}N$	Rb (μg/g)	$\delta^{15}N$	Rb (μg/g)
Oak leaves	3,310	948	19.3 (16)	4.2 (0.6)
Oak stems	2,010	366	13.9 (4.4)	5.5 (0.9)
Oak tap roots	93	32	1.7 (0.4)	4.2 (0.8)
Oak fine roots	56	19	1.1 (0.4)	−0.3 (0.5)
Grass shoots	NA	NA	4.2 (1.2)	−2.4 (1.4)

Values in parentheses are one standard error of the mean; *NA* no data. From Meding (2007)

Meding 2007). Donor oak leaves were labeled with RbCl and $K^{15}NO_3$. Receiver oaks were harvested 2 weeks later. About 25 % of receiver oaks contained both Rb and ^{15}N, indicating that hyphae had transferred the nutrients through the mesh and across the air gap (Table 3). Mean transfer was 30 μg ^{15}N and 34 μg Rb per seedling (Meding 2007). The amount of nutrients transferred did not correlate with distance between donor and receiver oak seedlings.

5.4 Plant Phenology and Nitrogen Acquisition

Plant phenology alters N uptake and transfer, with increased transfer when plants are growing rapidly. In a northern California blue oak-grassland, ^{15}N-urea was applied to beard grass (*Polypogon monspeliensis*) shoots for 2 days at times corresponding to three phenological stages: vegetative, flowering, and seed filling (Stout 2004). Two weeks after labeling the grasses, nearby seedlings of blue oak (*Q. douglasii*) and grasses and forbs were harvested. At the vegetative stage, N moved to neighboring grasses, forbs, and oak seedlings (Fig. 2). Nitrogen accumulated in shoots of forbs and grasses, but half of the newly acquired N accumulated in roots of oak. Seed-filling grass donors did not increase N transfer to neighboring plants, but retained N in their shoots. There were no preferential transfers of N from grass donors to grass receivers, suggesting that transfer did not occur via AM networks that link grasses. Although we cannot tell whether transfer occurred *directly* through a mycorrhizal network or *indirectly* by root or hyphal uptake from the soil, clearly, transfer was strongly sink driven and was influenced by the phenological stage of the donor grass (Stout 2004).

6 Carbon: Nutrient Exchange

Host plants transfer C to EM fungi, while fungi transfer nutrients to host plants. These transfers are affected by soil conditions (microbial activity and soil physical and chemical properties), plant conditions (phenology and plant age), and fungal

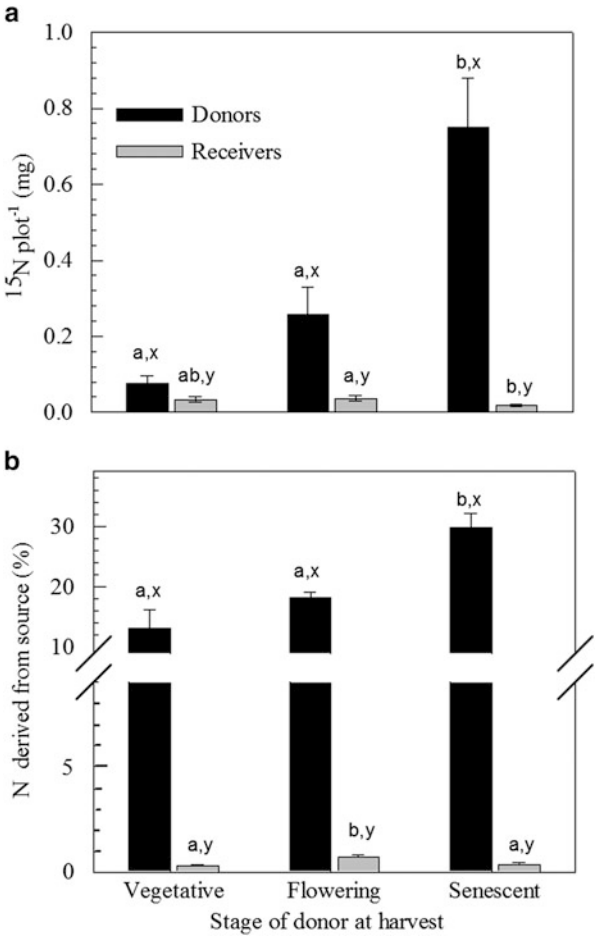

Fig. 2 More ^{15}N was transferred from donor grasses to receiver plants when grasses were vegetative or flowering and less was transferred when grasses were senescent: (**a**) mg^{15}N per plot, (**b**) percent N derived from source. Values are means (donor $n = 6$, receivers $n = 36$) ± standard errors. Different letters indicate significant differences ($p < 0.05$) among phenological stages for donors or receivers (**a**, **b**), and between donors and receivers within a stage (x, y). From Stout (2004)

conditions (AM and EM diversity, activity, and specificity for plant partners). While evidence indicates that this interaction is largely mutualistic, whether the exchange of resources is equally beneficial to EMs and to hosts remains unclear. If there is cooperation in the rhizosphere, do some fungi benefit more than others? Perhaps there are mycorrhizal "free riders" that obtain C from their host while providing few nutrients in return (Denison et al. 2003). Is there a continuum of mycorrhizal fungi from "cheaters" to "cooperators"? Answers to these questions are difficult to obtain due to the necessity to measure simultaneous fluxes of C and nutrients when fluxes are often multidirectional.

In a field study to measure C and N fluxes in foothill pine (*Pinus sabiniana*) saplings, stable isotopes of ^{13}C were applied to needles and ^{15}N to the soil (N) (Albarracin 2011). Some plots were fertilized with KNO_3 2 weeks before ^{15}N application. One month after ^{15}N addition, $^{13}CO_2$ was applied to bags around pine branches. Periodically (3–35 days) after labeling, needles and soil cores were collected for stable isotopic analyses. EM morphotypes were sorted by

color: black, brown, red, gold, tan, and "other." Descriptions of morphotypes suggested the following general identifications: black, *Cenococcum*; brown, Boletoid type; red, Thelephoroid type; and gold, *Hebeloma* type (Albarracin 2011). Enrichment for both ^{13}C and ^{15}N was greater for the gold morphotype compared to other morphotypes. The ^{13}C:^{15}N ratios (mean 5:1) varied by EM morphotype. Some morphotypes transferred more N per unit C received, benefitting the host more than other EM. These functional differences can affect host nutrition. In this same study, N fertilization increased ^{13}C transfer from host to EM but reduced ^{15}N transfer to hosts. EMs retained ^{15}N in hyphae and roots. Therefore N and C gain for EMs was enhanced by N fertilization at the expense of host pines, which lost C and received less N. These results challenge the idea that EM relationships are purely mutualistic. Instead the costs and benefits of mycorrhizal symbioses change with EM fungal species and with soil resources. Thus certain morphotypes were more "costly" for the host since they received more C but transferred less N, an example of a less mutualistic relationship. Other morphotypes were less "costly" and transferred more N per unit C received.

7 Lifespans and Dynamic Interactions

One of the challenges of studying mycorrhizal network in the field is the complex direction of resource movement in response to growth and mortality of the fungal hyphal networks, in contrast to root tips and rhizomorphs. One assumption from laboratory studies is that fungal hyphae are short-lived, although Orlov (1957) found EMs that lived 8–12 years. With the advent of minirhizotrons, we have begun to measure life spans of EMs in ecosystems and among fungal taxa. The results are surprising. Based on minirhizotron imagery, the life span of EM root tips averages 1 year, but some live for multiple years (Ruess et al. 2003; Allen et al. 2010; Kitajima et al. 2010). Based on ^{14}C from atmospheric bomb carbon, variability in hyphal age was associated with both species composition and soil conditions (Treseder et al. 2004). The age of individual mycorrhial root tips ranged from 1 to 6 years, with most between 4 and 5 years. Ectomycorrhizae of *Cenococcum* were comprised of younger carbon in N-amended plots than in controls. Importantly, sporocarp carbon came from new carbon.

The life spans of other fungal structures are less well known. The average rhizomorph life span was 11 months (Treseder et al. 2005). This means that most rhizomorphs would survive a growing season plus a drought period, remaining active and respiring (K. Kitajima and M.F. Allen, unpublished data). With high-resolution automated minirhizotrons (Allen et al. 2007), we have observed rhizomorphs and individual hyphal life spans in greater detail. Individual hyphae range in age from a few hours to several months, with the average age 46 days, longer than expected (M.F. Allen, unpublished data). This implies that the mycorrhizal network is highly dynamic, but not so dynamic that it cannot play a critical role in the exchange of resources among individual plants.

8 Creating a Dynamic and Complex Community

The goal of an organism is to survive through time. Numerous treatises studying mycorrhizae have noted that growth rates are not necessarily good indices of fitness, the ultimate measure of success or failure of an organism. But, what is an organism? The study of clonal organisms has confounded the study of plant communities because a clone of an organism, such as an aspen, may extend across large areas and exist for hundreds if not thousands of years. Mycorrhizal fungi expand that confounding issue to every terrestrial community. Is an organism a single 1 N nucleus with a defined genetic code? Or, a single, connected clone with genetically varied nuclei within the same connected body? Or, an organism (an AM fungus) that reproduces asexually, potentially for millions of years? In spite of these conceptual limitations, we continue to use the language of "organisms" knowing that our understanding of the relationship may need to be revised. If we can't define an organism, how are we to study and define a community of plants with their mycorrhizal fungi?

In the classical approach, a community is comprised of those organisms living in a given area in which members are measured as a unit, with known richness, diversity (using a variety of indices), and equitability. In a richer view, a community is comprised of organisms that influence each other's fitness (MacMahon et al. 1979). From this perspective, in terrestrial communities with mycorrhizal plants, no two plants neighboring each other fail to affect each other's fitness because each plant supports a hyphal network that likely links to its neighbor via interspecific direct connections, if only for a short time. Each hyphal network extends outward in an interconnecting web that potentially extends until an aquatic or disturbance barrier.

Therefore, understanding fitness of the differing fungi and plants from a point of origin to a barrier beyond which roots and hyphae no longer interact becomes a crucial topic that underlies understanding of the ecology of mycorrhizae and, in turn, plant communities. In perennial grasses, mycorrhizae enhanced plant survival and growth (and presumably fitness) through time mostly by improving root growth and development, which, over the life span of a perennial grass, would enhance fitness, particularly during stress years. Alternatively, in annual grasses, mycorrhizae enhanced seed production, the most critical attribute of an annual plant (Nelson and Allen 2006). How does the network of associated mycorrhizal fungi respond in the broader community? In tilled soils planted to annual crops, the types of mycorrhizal fungi differ from those in the surrounding perennial communities (Allen and Boosalis 1983). In a perennial community, plant and fungal responsiveness ranges from highly positive to minimal and even to negative responsiveness.

Importantly, the components of mycorrhizae—hyphae, mycorrhizal root tips, and rhizomorphs—have very different life spans that make the entire community dynamic. Each of these components could behave very differently in pot competition and in field studies in which the complex community of plants and fungi

continually interact. For these reasons, we need to initiate long-term field studies in which dynamics are monitored and modeled. From the perspective of mycorrhizal plant community-scale interactions, we have barely scratched the surface of our understanding.

9 Conclusions

The biocomplexity of the soil–plant–fungal matrix extends beyond mutualism. Mycorrhizal plants interact within a spatially and temporally variable soil environment and exchange soil nutrients and water as well as plant-derived carbon. These complex interactions vary in their costs and benefits to host plants and to mycorrhizal fungal partners. We have examined oak woodland ecosystems from Southern California to Southern Oregon and have a greater understanding of the properties that define biocomplexity. The AM and EM fungi differ in structural and biochemical properties including hyphal growth patterns, plant specificity, and enzymatic capabilities. Both AM and EM mycorrhizal networks form and interact—directing flows of water and nutrients in response to plant, fungal, and seasonal factors. Materials (C, N, P, and water) are exchanged between plants through these networks. Reciprocity of nutrient exchange between plant and fungus is not assured. Plants do not necessarily reward nutrient-efficient mycorrhizal fungi with more carbon. Overall, these dynamic interactions among mycorrhizal networks can help us unravel the complex relationships and the evolutionary histories that result in community and ecosystem dynamics.

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Ecology of Phreatophytes

Frank M. Thomas

Contents

Abstract Phreatophytes are plants with temporary or permanent access to groundwater. Despite distinct interspecific differences, many phreatophytes share the following traits: (1) rapid vertical root growth; (2) vigorous vegetative regeneration; (3) a significant fraction of xylem conduits with large diameters; (4) high hydraulic conductance; (5) relatively high vulnerability to xylem embolism; (6) high foliar conductance to water vapour; (7) high rates of biomass production; and (8) low water use efficiency. Phreatophytes can be considered a hydro-ecological plant type, and put in a line with hydrophytes, hygrophytes, and xerophytes. In subdividing phreatophytes according to their dependence on groundwater, it is suggested to replace the conventional term "obligate" with "permanent", and "facultative" with "temporary" as the fraction of the water demand that is covered by uptake of groundwater seems to be determined by environmental conditions rather than by inherent traits of the plants.

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1 Phreatophytes: Definition, Distribution, and Identification

The term "phreatophyte" derives from the classical Greek word "φρεαρ" (well, cistern). Daly (1917) used the term "phreatic water" as a synonym for groundwater for all water in the zone of water saturation. Meinzer (1923) coined the term "phreatophyte" for "a plant that habitually obtains its water supply from the zone of saturation, either directly or through the capillary fringe". In this sense, it has been used ever since in ecological literature.

In most probability, phreatophytic plants have already occurred in the Triassic. This has been concluded from the discovery of carbonate cementation in sandstone found in Southwestern England, which was interpreted as excretions from tap roots of phreatophytes (Purvis and Wright 1991). In human history, the phenomenon that some plants are capable of meeting their demand of water by directly tapping the water table is known to mankind since many centuries. In parables of the Old Testament, Ross (2007) found descriptions of typical features of phreatophytes.

Phreatophytic characteristics can be considered to be adaptations to the dry site conditions of arid or semi-arid regions. In regions where during longer periods of the growing season, precipitation or inundation is insufficient to sustain the survival and growth of the plants, phreatophytes generally have an advantage in competition at sites where the water table is not too deep to be reached by roots. Consequently, phreatophytes typically can be found in arid and semi-arid regions along rivers, streams, washes or lakeshores, on floodplains, in grassland and deserts (including oases), in dry heathland, shrub- and woodland, but also on coastal sand dunes. In some regions, phreatophytic vegetation contributes to shaping the landscape; this is the case for the riparian (*tugai*) forests of Central Asia (Walter and Box 1983; Kürschner 2004; Fig. 1) and the *Prosopis* woodland of the Monte Desert in Argentina (Mares et al. 1985). Phreatophytes can be found in all continents except Antarctica. Several studies on phreatophytes have been conducted in America and Asia, but only a small number in Africa. From these figures, however, one cannot conclude on the abundance of phreatophytic species in those regions because of the bias in the number of research projects funded and conducted in the respective countries. Table 1 provides an overview of the distribution and site characteristics of phreatophytic species that have been covered in recent literature and by some older sources. In most probability, this list is not complete, which is also due to the fact that for some species such as *Welwitschia mirabilis* it is not unambiguously clear whether it is a phreatophyte or not (Veste 2004).

Besides groundwater, water from precipitation or run-off that seeps through rock fractures and eventually accumulates in bedrock voids (cf. Evenari 1985) can constitute an important water supply not only for animals, but also for perennial plants whose roots are capable of tapping this water. One example is the shrub *Malosma laurina* (Nutt.) Abrams (syn. *Rhus laurina* Nutt.; Anacardiaceae), which grows at slopes and in canyons of the South Californian chaparral and is capable of sending its roots deeper than 13 m into the soil (DeSouza et al. 1986). Although such species are no phreatophytes in the term's true sense and, thus, are not covered

Fig. 1 *Populus euphratica* woodland with *Tamarix ramosissima* understorey in the transition zone between a river oasis and the open desert at the southern fringe of the Taklamakan Desert, Northwestern China, in vicinity to an old river bed. The distance between the canopy of *P. euphratica* and the groundwater was as large as 23 m (Gries et al. 2003) due to sand accumulation after the establishment of the trees. The height growth of the trees could keep pace with the sand accumulation. Thus, a large part of the stems of those trees that resulted from establishment is now buried by sand (photograph: F.M. Thomas)

in the present review, they are equally remarkable in their capability to exploit water resources, and comparisons of their ecology with that of "true" phreatophytes deserve future research.

Almost all plant families that include phreatophytic species belong to the eudicot subclass of the angiosperms. Some few Poacean and Arecacean species (e.g. *Phoenix dactylifera*) that are part of the monocot subclass are exceptions. Several eudicot genera such as *Acacia*, *Atriplex*, *Banksia*, *Calligonum*, *Eucalyptus*, *Haloxylon*, *Nitraria*, *Populus*, *Prosopis*, *Quercus*, *Salix*, and *Tamarix* even contain two or more phreatophytic species. No phreatophytes are known that would belong to the angiospermous "basal orders" (formerly: Magnoliidae). In these orders, the lack of phreatophytic species possibly is due to the prevalence of wood with primal features such as conduits with relatively small diameters and scalariform perforation plates, which have a higher resistance to water flow than conduits with larger diameters and simple perforation plates (cf. Dickison 2000; Carlquist 2001). However, more investigations of the functional wood anatomy and the occurrence of "Magnoliid" taxa in specific environments would be necessary to support this hypothesis.

For annual plants of arid and semi-arid regions, the supply of meteoric water can be sufficient to complete their life cycle. However, plants can take fundamental competitive advantage if they are capable of tapping groundwater. If the distance to the water table is larger than only some few decimetres, they have to develop a sturdy tap root for the continuous uptake of water. Accordingly, most phreatophytes are perennial woody species (trees, shrubs, or sub-shrubs) of phanerophytic or chamaephytic Raunkiaer life form (Table 1). Only few phreatophytic species (e.g. *Distichlis spicata*,

Table 1 Phreatophytic species and their plant family, growth and life form, distribution, habitat, conventional subdivision into facultative or obligate phreatophyte, distance to groundwater, and selected references

Species	Plant family	Growth form	Raunkiaer life form	Distribution	Habitat	Obligate/ facultative	Distance to ground water (m)	References (selection)
Acacia auriculiformis A. Cunn. ex Benth.	Fabaceae	Shrub/tree	Phanerophyte	North Australia	Riparian woodlands, streambanks	Facultative	3.5	Lamontagne et al. (2005)
Acacia erioloba E. Meyer	Fabaceae	Tree	Phanerophyte	Southern Africa	Sandy soils of desert plains, riverbeds, streambanks	n.i.	60	Jennings (1974), cited by Canadell et al. (1996)
Acacia tortilis (Forssk.) Hayne	Fabaceae	Shrub/tree	Phanerophyte	North Africa, Middle East	Woodlands, savanna	Facultative	≤30 m	Wickens (1998), Stave et al. (2005)
Acanthosicyos horridus Welw. ex Hook. fil	Cucurbitaceae	Shrub	Chamaephyte	Namibia, coast of Namib Desert	Dry riverbeds, sand dunes	Obligate	8–12	Klopatek and Stock (1994)
Acer negundo L.	Sapindaceae	Tree	Phanerophyte	North America to mountains of Mexico	Riparian and palustrine habitats	Facultative	n.i.	Kolb et al. (1997)
Alhagi sparsifolia Shap.	Fabaceae	Sub-shrub	Chamaephyte	Central Asia (Kazakhstan, Uzbekistan, Turkmenistan to Mongolia; Xinjiang, Gansu)	Desert plains	Obligate (?)	≤20	Thomas et al. (2006a), Bruelheide et al. (2010), Vonlanthen et al. (2010b)
Allenrolfea occidentalis	Chenopodiaceae	Sub-shrub/ shrub	Chamaephyte	Southwestern North America	Sandy hummocks	Facultative	≤6	

(S. Watson) Kuntze					in salt playas and mud flats			Nichols (1994), Trent et al. (1997)
Artemisia tridentata Nutt.	Asteraceae	Shrub	Phanerophyte	North America	(Semi-)arid shrubland, neutral to alkaline soils	Facultative	3.7	Nichols (1994)
Atriplex canescens (Pursh.) Nutt.	Chenopodiaceae	Shrub	Phanerophyte	Western North America; Northern and Central Mexico	Sand dunes, deserts	Facultative	≤19	Robinson (1958), Nichols (1994), McKeon et al. (2006)
Atriplex confertifolia (Torr. and Frem)	Chenopodiaceae	Shrub	Phanerophyte/ chamaephyte	Southwestern North America; Northern Mexico	Warm and cold saline deserts	Facultative	1.8–5.5	Nichols (1994)
Atriplex gardneri (Moq.) D. Dietr. var. tridentata (Kuntze) Macbr.	Chenopodiaceae	Sub-shrub	Chamaephyte	Western North America	Saline, arid, nutrient-poor soils	Facultative (?)	n.i.	Nichols (1994)
Banksia attenuata R. Br.	Proteaceae	(Shrub)/ tree	Phanerophyte	Western Australia	Sand dunes, sandplains	Facultative	6–>30	Dodd and Bell (1993a), Zencich et al. (2002), Canham et al. (2009)
Banksia ilicifolia R. Br.	Proteaceae	Tree	Phanerophyte	Southwestern Australia	Deep sand in shrub- and woodland	Obligate/ facultative	<8	Zencich et al. (2002), Froend and Drake (2006)
Banksia littoralis R. Br.	Proteaceae	Shrub/tree	Phanerophyte	Southwestern Australia	Streambanks, moist sites	Obligate	≤3.2	Canham et al. (2009)
Banksia menziesii R. Br.	Proteaceae	(Shrub)/ tree	Phanerophyte	Western Australia	Sand dunes, sandplains	Facultative	6–>30	

(continued)

Table 1 (continued)

Species	Plant family	Growth form	Raunkiaer life form	Distribution	Habitat	Obligate/ facultative	Distance to ground water (m)	References (selection)
								Dodd and Bell (1993a), Canham et al. (2009)
Banksia prionotes Lindl.	Proteaceae	Shrub/tree	Phanerophyte	Southwestern Australia	Sand dunes, sandplains	Facultative	1.8–3.8	Pate et al. (1995), Dawson and Pate (1996)
Barringtonia acutangula (L.) Gaertn.	Lecythidaceae	Shrub/tree	Phanerophyte	Northern Australia	Streambanks	Obligate	5	Lamontagne et al. (2005)
Boscia albitrunca (Burch.) Gilg and Ben. var. albitrunca	Capparaceae	Tree	Phanerophyte	South Africa	Streambanks	Facultative	68	Jennings (1974), cited by Canadell et al. (1996), Wand et al. (1999)
Calligonum spp.	Polygonaceae	Shrub	Phanerophyte	Mediterranean region, Asia	Steppes, sand dunes	Facultative or obligate	ca 12 m in *C. caput-medusae*	e.g., Thomas et al. (2006a)
Carya illinoensis (Wangenh.) K. Koch	Juglandaceae	Tree	Phanerophyte	North America (Mississippi River valley)	Alluvial soils, well-drained flats	Facultative	7	Sparks (2005)
Cathormion umbellatum (Vahl.) Kosterm.	Fabaceae	Shrub/tree	Phanerophyte	North Australia	Streambanks, monsoon forests	Facultative	7	Lamontagne et al. (2005)
Celtis reticulata Torr.	Ulmaceae	Tree	Phanerophyte	Western North America;	Riparian woodlands and forests	Facultative	≤4.1	Hultine et al. (2003)

				Northern Mexico				
Chilopsis linearis (Cav.) Sweet	Bignoniaceae	Shrub/tree	Phanerophyte	Southwestern North America; Northern Mexico	Streambanks	Obligate	n.i.	de Soyza et al. (2004)
Chrysothamnus nauseosus (Pall.) Britton (syn. *Ericameria nauseosa* (P. von Pall. ex F. Pursh.) G. Nesom and G. Baird)	Asteraceae	Shrub	Phanerophyte	Western North America; Northern Mexico	(Semi-)arid scrub- and woodland, salt deserts	Obligate/ facultative	1.1–10.5	Robinson (1958), Nichols (1994), Chimner and Cooper (2004), Kray et al. (2012)
Citrullus colocynthis L. (Schrad.)	Cucurbitaceae	Herb	Hemicrypto-phyte	Mediterranean region, North Africa, Middle East, South-western Asia	Sand and gyp-sum desert, sandy gravel	Obligate	n.i.	Althawadi and Grace (1986)
Distichlis spicata (L.) Greene	Poaceae	Grass	Hemicryptophyte	North America	Tidal marshes, riparian habitats, grasslands, desert	Facultative	1.1 to ≤3.6	Nichols (1994), Steinwand et al. (2006), Kray et al. (2012)
Elaeagnus angustifolia Hill.	Elaeagnaceae	Shrub/tree	Phanerophyte	Eastern Europe to Northern China	Sea shores, river and lake shores, dry river beds	Obligate (?)	n.i.	Zeng et al. (2006)

(continued)

Table 1 (continued)

Species	Plant family	Growth form	Raunkiaer life form	Distribution	Habitat	Obligate/ facultative	Distance to ground water (m)	References (selection)
Eremaea pauciflora (Endl.) Druce	Myrtaceae	Shrub	Phanerophyte	Western Australia	Sandplains	Facultative	6–7	Dodd and Bell (1993a)
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Tree	Phanerophyte	Southeastern to southwestern Australia	Streambanks, floodplains	Facultative	2–3	Thorburn and Walker (1994)
Eucalyptus gomphocephala D.C.	Myrtaceae	Tree	Phanerophyte	Southwestern Australia	Coastal plains	n.i.	≤8.0	Drake et al. (2011)
Eucalyptus marginata Sm.	Myrtaceae	Tree	Phanerophyte	Southwestern Australia	Sandy to clayey soils	n.i.	ca 20	Carbon et al. (1980)
Euclea pseudebenus E. Mey. ex A. DC.	Ebenaceae	Tree	Phanerophyte	South Africa	Streambanks	Facultative	n.i.	Wand et al. (1999)
Faidherbia albida (Del.) A. Chev. (syn. *Acacia albida* Del.)	Fabaceae	Tree	Phanerophyte	Southern Africa, Middle East	Streambanks	Facultative	≥40	Roupsard et al. (1999), Leduc et al. (2001)
Fraxinus velutina Torr.	Oleaceae	Tree	Phanerophyte	Southwestern North America	Wetlands, riparian habitats	Facultative	≤4.1	Hultine et al. (2003)
Grayia spinosa (Hook.) Moq.	Chenopodiaceae	Shrub	Phanerophyte	Western North America	Desert shrub- and woodland	Facultative	≤5.8	Nichols (1994)
Halocnemum strobilaceum (Pall.) M. Bieb.	Chenopodiaceae	Sub-shrub	Hemicrypto-phyte	Mediterranean region, Arabia to Central Asia and Mongolia	Salt marshes, salt dunes	n.i.	n.i.	Guvensen and Ozturk (2003), Al-Dousari et al. (2008)
	Amaranthaceae	Small tree	Phanerophyte	Central Asia		Facultative	≤20 m	Wei et al. (2008)

Haloxylon ammodendron (C. A. Meyer) Bunge					Sandy areas in saline-alkaline deserts			
Haloxylon aphyllum (Minkw.) Iljin	Amaranthaceae	Shrub/ Small Tree	Phanerophyte	Middle and Central Asia	Dry, sandy to loamy, saline sites	Facultative	≤16 m	Walter and Breckle (1994), Gintzburger et al. (2003)
Jacksonia floribunda Endl.	Fabaceae	Shrub	Phanerophyte	Western Australia	Lateritic hills, sandplains	Facultative	6–7	Dodd and Bell (1993b)
Juglans major (Torr.) Heller	Juglandaceae	Tree	Phanerophyte	Southwestern North America; Northern Mexico	Streambanks, floodplains	Facultative	≤4.1	Hultine et al. (2003)
Juniperus monosperma (Engelm.) Sarg.	Cupressaceae	Tree	Phanerophyte	Southwestern North America	Desert grassland, pinyon-juniper woodland	n.i.	>61	Cannon and Starrett (1956), cited after Stone and Kalisz (1991)
Karelinia caspia (Pall.) Lessing	Asteraceae	Shrub	Phanerophyte	Iran, Turkey, Russia, Mongolia, Central Asia, Gansu	Gobi Desert, dunes, saline meadows	Obligate (?)	≤14	Bruelheide et al. (2010)
Krascheninnikovia ceratoides (L.) Gueldenst. (syn. *Eurotia ceratoides* (L.) C. A. Meyer	Chenopodiaceae	Sub-shrub	Chamaephyte	Middle and Central Asia	Desert steppe, alpine shrubland, sandy loam to rocky debris	n.i.	n.i.	Wickens (1998)
Krascheninnikovia lanata (Pursh.) A. D. J. Meeuse and Smit (syn. *Eurotia lanata* (Pursh.) Moq.)	Chenopodiaceae	Shrub	Phanerophyte	Western North America; Northern Mexico	Arid to semi-arid, saline shrubland	Facultative	n.i.	Nichols (1994)

(continued)

Table 1 (continued)

Species	Plant family	Growth form	Raunkiaer life form	Distribution	Habitat	Obligate/ facultative	Distance to ground water (m)	References (selection)
Melaleuca argentea W. Fitzg.	Myrtaceae	Tree	Phanerophyte	North Australia	Riparian forests, streambanks	Obligate	3	Lamontagne et al. (2005)
Nitraria retusa (Forssk.) Aschers.	Nitrariaceae	Shrub	Phanerophyte	Sahara, Mediterranean region, Middle East	Salt deserts	n.i.	n.i.	Wickens (1998), Al-Dousari et al. (2008)
Nitraria tangutorum Bobrov	Nitrariaceae	Shrub	Phanerophyte	NW China	Sandy areas, clay deserts, semi-deserts	Facultative	≤20	Kürschner (2004), Wei et al. (2008), Zhu et al. (2011)
Ozoroa concolor (Presl ex Sond.) DeWinter	Anacardiaceae	Tree	Phanerophyte	South Africa	Streambanks	Facultative	n.i.	Wand et al. (1999)
Phoenix dactylifera L.	Arecaceae	Tree	Phanerophyte	Mediterranean region, North Africa, Southwestern Asia	Surroundings of permanent desert springs	Obligate	n.i. (small as *P. d.* is shallow-rooted)	Gibson (1996), Wickens (1998)
Phragmites australis Trinius ex Steudel	Poaceae	Grass	Hemicryptophyte	Cosmopolitan	River banks, lake shores	Facultative	≤8	Thevs et al. (2008b), Bruelheide et al. (2010)
Pluchea sericea (Nutt.) Cov.	Asteraceae	Shrub	Phanerophyte	Southwestern North America	Riparian habitats	Facultative	0.5–1	Sala et al. (1996)
Populus angustifolia James	Salicaceae	Tree	Phanerophyte	Western North America	Riparian areas	Obligate/ facultative	≤1.4–2.3	Letts et al. (2008), Rood et al. (2011)
	Salicaceae	Tree	Phanerophyte			Facultative	≤1.2	Rood et al. (2011)

Populus balsamifera L. ssp. balsamifera L.				Central and Northern North America	Riparian areas of boreal/montane conifer forests			
Populus balsamifera L. ssp. trichocarpa (Torr. and A. Gray ex Hook.) Brayshaw	Salicaceae	Tree	Phanerophyte	Western North America	Streambanks	Facultative	≤0.7	Rood et al. (2011)
Populus deltoides Marsh.	Salicaceae	Tree	Phanerophyte	Southern North America	Streambanks	Facultative	<2	Cleverly et al. (2006), Rood et al. (2011)
Populus euphratica Oliv.	Salicaceae	Tree	Phanerophyte	Easternmost Mediterranean region; Middle East to China	Riparian floodplain	Obligate	≤23	Gries et al. (2003), Thomas et al. (2006a), Bruelheide et al. (2010), Vonlanthen et al. (2010b)
Populus fremontii S. Wats.	Salicaceae	Tree	Phanerophyte	Southwestern North America; Northern Mexico	Riparian floodplain	Obligate/facultative	1.2 to ≤5	Busch et al. (1992), Schaeffer et al. (2000), Shafroth et al. (2000), Snyder and Williams (2000), Horton et al. (2001a)
Prosopis alpataco Phil.	Fabaceae	Shrub/tree	Phanerophyte	Bolivia, Argentina	Grassland	Facultative	n.i.	Villagra and Junent (1997)
	Fabaceae	Tree	Phanerophyte			Obligate (?)	≤36	Wickens (1998)

(continued)

Table 1 (continued)

Species	Plant family	Growth form	Raunkiaer life form	Distribution	Habitat	Obligate/ facultative	Distance to ground water (m)	References (selection)
Prosopis cineraria (L.) Druce				Arabia to Pakistan and India	Dry deciduous and thorn forest, scrubland, desert dunes			
Prosopis flexuosa D. C.	Fabaceae	Tree	Phanerophyte	Argentina, Bolivia, Chile	Arid woodland	Facultative	6–15	Guevara et al. (2010), Giordano et al. (2011), Jobbágy et al. (2011)
Prosopis glandulosa Torr.	Fabaceae	Shrub/tree	Phanerophyte	Southwestern North America; Northern Mexico	Oak woodland, drainageways, riparian habitats	Facultative	≤9	Nilsen et al. (1983), Laity (2003)
Prosopis pallida (Humb. and Bonpl. ex Willd.) Kunth	Fabaceae	Shrub/tree	Phanerophyte	Ecuador, Peru	Arid woodland	Facultative	≤25	López et al. (2005)
Prosopis pubescens Benth.	Fabaceae	Shrub/tree	Phanerophyte	Southwestern North America; Northern Mexico	Riparian woodlands and scrubs	n.i.	0.5–1.0	Sala et al. (1996)
Prosopis tamarugo Phil.	Fabaceae	Tree	Phanerophyte	Chile	Warm temperate and subtropical desert, thorn steppe, subtropical thorn forest	Obligate	≤12	Mooney et al. (1980), Alonso (1990), Houston (2006)
Prosopis velutina Woot.	Fabaceae	Shrub/tree	Phanerophyte	Southwestern North	Grassland, riverbanks	Facultative	10	

				America; Northern Mexico				Scott et al. (2000), Snyder and Williams (2000)
Quercus agrifolia Nee	Fagaceae	Tree	Phanerophyte	California, Northern Mexico	Oak woodland	Obligate (?)	n.i.	Griffin (1973)
Quercus douglasii Hook. and Arn.	Fagaceae	Tree	Phanerophyte	California	Oak woodland	Obligate	24	Lewis and Burgy (1964), cited by Stone and Kalisz (1991), Miller et al. (2010)
Quercus lobata Nee	Fagaceae	Tree	Phanerophyte	California	Oak woodland	Obligate (?)	n.i.	Griffin (1973)
Quercus wislizenii A. D.C.	Fagaceae	Tree	Phanerophyte	California	Riparian sites, wetlands	n.i.	24	Lewis and Burgy (1964), cited by Stone and Kalisz (1991)
Retama sphaerocarpa (L.) Boiss.	Fabaceae	Shrub	Phanerophyte	Northwestern Africa, Iberian Peninsula	(Semi-)arid shrubland	Facultative	>30	Domingo et al. (2003)
Rhus populifolia E. Mey. ex Sond.	Anacardiaceae	Shrub	Phanerophyte	South Africa	Streambanks	Facultative	n.i.	Wand et al. (1999)
Salix exigua Nutt.	Salicaceae	Shrub/tree	Phanerophyte	Western North America	Plains, lower elevations to lower montane habitats	n.i.	0.5–1.0	Sala et al. (1996)
Salix gooddingii C.R. Ball	Salicaceae	Tree	Phanerophyte	Southwestern North America	Streambanks	Obligate	1.2–≤5	Busch et al. (1992), Schaeffer et al. (2000)
Salix lemmonii Bebb	Salicaceae	Shrub	Phanerophyte			n.i.	n.i.	

(continued)

Table 1 (continued)

Species	Plant family	Growth form	Raunkiaer life form	Distribution	Habitat	Obligate/ facultative	Distance to ground water (m)	References (selection)
				Western North America	Streambanks, floodplains, riparian habitats			Svejcar and Trent (1995)
Salix monticola Bebb	Salicaceae	Shrub	Phanerophyte	Southwestern North America	Streambanks, moist slopes; southern distribution area: >1,800 m	n.i.	n.i.	Foster and Smith (1991)
Salix repens subsp. *dunensis* Rouy	Salicaceae	Shrub	Chamaephyte	Baltic States, Central/Northern Europe, British Isles	Coastal dunes	Facultative	n.i.	Máguas et al. (2011)
Sarcobatus vermiculatus (Hook.) Torr.	Chenopodiaceae	Shrub	Phanerophyte	Western North America; Northern Mexico	Wetlands to cold deserts, mostly on more or less saline soils	Facultative	1 to ≤13.0	Chimner and Cooper (2004), McKeon et al. (2006), Kray et al. (2012)
Schotia afra (L.) Thunb. var. angustifolia (E. Mey.)	Fabaceae	Tree	Phanerophyte	South Africa	Streambanks	Facultative	n.i.	Wand et al. (1999)
Spartidium saharae (Coss. and Reboud) Pomel	Fabaceae	Shrub	Chamaephyte/ phanerophyte	North Africa	Hot desert	n.i.	n.i.	Abdallah and Chaieb (2007)
Sporobolus airoides (Torr.) Torr.	Poaceae	Grass	Hemicryptophyte	Western North America;		Facultative	1.1 to ≤3.0	

				Northern Mexico	Saline and non-saline grassland			Steinwand et al. (2006), Kray et al. (2012)
Stirlingia latifolia (R. Br.) Steud.	Proteaceae	Shrub	Phanerophyte	Western Australia	Heath, shrubland, woodland	Obligate (?)	6–7	Dodd and Bell (1993a)
Styrax officinalis L. var. *redivivus* (Torrey) H. Howard	Styracaceae	Shrub	Phanerophyte	Southern Europe, Middle East, California	(Semi-)arid shrubland	Facultative	n.i.	Mahall et al. (2010)
Tamarix aphylla (L.) H. Karst.	Tamaricaceae	Shrub/tree	Phanerophyte	North Africa to India	Sand, loess and salty soils, dunes, river beds	n.i.	>10 m	Wickens (1998)
Tamarix chinensis Lour. (Ledeb.)	Tamaricaceae	Shrub	Phanerophyte	Asia; naturalised in Western North America	Riparian habitats	Facultative	≤7.8	Horton et al. (2001b), Cleverly et al. (2006)
Tamarix gallica L.	Tamaricaceae	Shrub	Phanerophyte	Western Mediterranean and Sub-Mediterranean region	Riverbanks, sea shore	Facultative	≤2.7	Mounsif et al. (2002)
Tamarix ramosissima Ledeb.	Tamaricaceae	Shrub	Phanerophyte	Temperate zones of Asia, invasive in North America	Riparian floodplain	Facultative	0.5–24	Sala et al. (1996), Gries et al. (2003), Vonlanthen et al. (2010b)
Tamarix usneoides E. Mey. ex Bunge	Tamaricaceae	Tree	Phanerophyte	South Africa	Streambanks	Facultative	n.i.	Wand et al. (1999)
	Asteraceae	Shrub/tree	Phanerophyte			Facultative	3.6	

(continued)

Table 1 (continued)

Species	Plant family	Growth form	Raunkiaer life form	Distribution	Habitat	Obligate/ facultative	Distance to ground water (m)	References (selection)
Tessaria sericea (Nutt.) Shinners				Southwestern North America	Riparian floodplain			Busch and Smith (1993)
Ulmus pumila L.	Ulmaceae	Tree	Phanerophyte	Northern China, East Siberia, Mongolia, Korea	Riparian woodland	Obligate	n.i.	Kürschner (2004)
Zygophyllum prismatocarpum E. Mey. ex Sond.	Zygophyllaceae	Shrub	Phanerophyte	South Africa	Streambanks	Facultative	n.i.	Wand et al. (1999)

n.i. no information provided; (?), unclear

Phragmites australis, and *Sporobolus airoides*; Table 1) are non-woody, hemicryptophytic grass species. In contrast, leaf longevity obviously is not closely related to the phreatophytic existence. Among phreatophytes, evergreen, cold-deciduous, drought-deciduous, and even rain-deciduous species (*Faidherbia albida*; Roupsard et al. 1999) occur. The shrubby phreatophyte *Acanthosicyos horridus* has no leaves; photosynthesis is mainly conducted by the green stems. Only some of the phreatophytic species bear succulent leaves (e.g. *Atriplex canescens*, *Karelinia caspia*, *Sarcobatus vermiculatus*); many phreatophytes are not succulent.

Phreatophytes have been regarded as a plant functional group or type (Sperry and Hacke 2002; Xu and Li 2006). However, the same criticism would apply to this attribution as has been raised against the concept of plant functional types in general: (1) besides belonging to the phreatophytes, phreatophytic species can at the same time be part of another functional group, which results in inconsistency of grouping; (2) phreatophytes are characterised by functional response traits as well as by functional effect traits, which renders the exact meaning of "functional type" unclear; and (3) often, functional response and functional effect traits are only gradually differentiated; this can make the grouping of a given species difficult or even impossible. Therefore, it can be presumed that introduction of phreatophytes as a specific functional group or type in ecological literature would not yield deeper insight into ecosystem functioning and processes.

Conventionally, phreatophytic species are subdivided into obligate phreatophytes, which rely upon continuous access to groundwater, and facultative phreatophytes, which have access to groundwater only during certain periods of the year (Laity 2008, p 248), and are also capable of using water that infiltrates from the soil surface. Thus, the vertical extent of their root systems into the soil can vary according to the water supply at the sites of their occurrence (e.g. Rood et al. 2011). However, an "obligate" phreatophytic behaviour seems to be more related to the environmental conditions than to the capabilities of a given plant species. Firstly, it is barely conceivable that "obligate" phreatophytes would not be capable of using water that infiltrates from the surface (provided that the soil water potential is not too negative, and that the water is available to the plant for a sufficient period of time). This has also been considered by Robinson (1958). Secondly, in some phreatophytic species (*Prosopis flexuosa*, Guevara et al. 2010; *Populus* spp., Rood et al. 2011), considerable phenotypic plasticity of the root system has been found in dependence upon the source of accessible water: within a given species, the plants formed a shallow and horizontal root structure at sites where water infiltrating from the surface could be taken up, and developed deeper-reaching vertical roots at drier sites where water was principally taken up from the water table or its capillary fringe. Such plasticity can only be detected when a species is investigated along a gradient of different water sources (infiltrating vs. groundwater), which has only scarcely been done with phreatophytes. Thirdly, this dependence on the site conditions can result in contrasting characterisations of a given phreatophytic species as "obligate" or "facultative", as was the case for *Banksia ilicifolia* (Zencich et al. 2002 vs. Froend and Drake 2006 and Canham et al. 2009), *Chrysothamnus nauseosus* (e.g. Nichols 1994 vs. Kray et al. 2012), *Populus angustifolia* (Letts et al. 2008 vs. Rood et al. 2011), and *P. fremontii* (Schaeffer et al. 2000 and Shafroth et al. 2000 vs. Snyder and Williams 2000 and Horton et al. 2001a).

Fourthly, the occurrence of obligate phreatophytic vegetation is not restricted to sites where the water table lies only some few metres below the soil surface; rather, this vegetation can also occur at sites with considerably larger distances to the water table. Therefore, it does not necessarily exhibit linear patterns along current river courses (Bruelheide et al. 2010); this renders the visual differentiation between obligate and facultative phreatophytes even more difficult. Therefore, it should be considered to replace the term "obligate" by "permanent", and the term "facultative" by "temporary", and to use these terms only in combination with the respective site conditions. Of course, as with the conventional terms, a reliable differentiation into "permanent" and "temporary" can only be made once a plant has established on the locality of its occurrence. Nevertheless, for a more complete characterisation of the species, and to make comparisons with the literature easier, the terms "obligate" and "facultative" are still provided in Table 1 and throughout the text.

Phreatophytes can be identified by (1) direct observation, e.g. at riverbanks that have been vertically cut by erosion (Rood et al. 2011); (2) comparison of the isotope ratios of ^{2}H or ^{18}O in plant water and groundwater (e.g. Zencich et al. 2002; Horton et al. 2003; Chimner and Cooper 2004; Kray et al. 2012); (3) comparison of the concentrations of chemical compounds in the plant and the groundwater (e.g. electrical conductivity of groundwater and extracts of leaves from *Prosopis velutina* Woot. [=*P. articulata* S. Watson], García-Carreño et al. 1992; concentrations of boron, bromine, sodium, and strontium in contaminated groundwater and leaves of *Populus deltoides*, Erdman and Christenson 2000); (4) circumstantial evidence using ecophysiological measurements of leaf water potential and plant transpiration in comparison to precipitation, corroborated by measurements of the soil water potential and records of the occurrence of fine roots in the non-saturated soil layers (Thomas et al. 2006a); and (5) circumstantial evidence on the basis of above-ground productivity of the phreatophytes in comparison to annual precipitation (e.g. Gries et al. 2005).

2 Adaptations of Phreatophytes to Environmental Factors

2.1 Germination, Establishment, Rooting Depth and Regeneration

Especially at (semi-)arid sites, germination and the establishment of the seedlings are challenging periods in the plant's life cycle. In the riparian vegetation of hyper-arid deserts (i.e. deserts with a ratio of mean annual precipitation to mean annual evapotranspiration of <0.03 mm mm^{-1}; Whitford 2002) such as the Taklamakan Desert in Northwestern China, germination can only take place at sites where the soil has been thoroughly wetted through flooding or inundation by rivers, and lack competing vegetation (Thevs et al. 2008a). After germination, the seedlings still are threatened by desiccation and can suffer high mortality. In *Populus deltoides* floodplains of arid Northwestern Colorado, the *Populus* seedlings did not reach the groundwater before the third or fourth vegetation period even in the locations

that were most favourable for establishment (Cooper et al. 1999). In addition, seeds of some phreatophytes, e.g. of *Tamarix* species, have only a short period of viability (Di Tomaso 1998). Therefore, successful establishment of phreatophyte seedlings often is restricted to only a few germination events. These can also be associated with climatic conditions. In northern Peru, which is severely affected by ENSO (El Niño Southern Oscillation) events, the establishment of *Prosopis pallida* seedlings was found to be much more successful during warm periods with simulated ENSO precipitation than in cooler North-Central Chile, which is much less influenced by ENSO events (Squeo et al. 2007).

Upon germination, phreatophytes must strive to send their roots into the water-saturated zone of the soil as rapidly as possible. Generally, this is achieved by forming a vertically growing tap root. In a field experiment including irrigation, the roots of seedlings of the sub-shrub *Alhagi sparsifolia* and the shrub *Karelinia caspia*, both widely distributed in Central Asia, reached down to a depth of 1.7 m and 2.2 m, respectively, after only 16 weeks; these high root growth rates enable the plants to reach groundwater tables, or to follow slowly declining groundwater tables, within one growing season (Vonlanthen et al. 2010a). High rates of vertical root growth (up to 0.8 m and 1.2 m, respectively, within 8 weeks) were also found in the tree species *Faidherbia albida* from South Africa and *Acacia tortilis* (North Africa, Middle East) (Stave et al. 2005). In several cases, woody phreatophytes that can grow as a shrub or small trees (e.g. *Haloxylon aphyllum*) only form large plants and dense vegetation once their roots have reached the groundwater (Fig. 2).

The majority of phreatophytic species do not seem to root very deeply in the soil. However, considerable rooting depths have been observed in some phreatophytes. On the basis of the present literature survey, I found 28 species for which rooting depths of more than 10 m have been recorded (*Acacia erioloba* and *A. tortilis*; *Acanthosicyos horridus*; *Alhagi sparsifolia*; *Atriplex canescens*; *Banksia attenuata* and *B. menziesii*; *Boscia albitrunca*; *Calligonum caput-medusae*; *Chrysothamnus nauseosus*; *Eucalyptus marginata*; *Faidherbia albida*; *Haloxylon aphyllum* and *H. ammodendron*; *Juniperus monosperma*; *Karelinia caspia*; *Nitraria tangutorum; Populus euphratica*; *Prosopis cineraria*, *P. flexuosa*, *P. pallida*, and *P. tamarugo*; *Quercus douglasii* and *Q. wislizenii*; *Retama sphaerocarpa*; *Sarcobatus vermiculatus*; *Tamarix aphylla* and *T. ramosissima*; Table 1). *Tamarix* species generally have deep-reaching roots (Baum 1978), but not for all these species, a phreatophytic nature is evident from the literature. Maximum rooting depths that exceeded 30 m were reached by the phreatophytes *Acacia erioloba*, *Banksia attenuata*, *B. menziesii*, *Boscia albitrunca*, *Faidherbia albida*, *Juniperus monosperma*, *Prosopis cineraria*, and *Retama sphaerocarpa*. This exceeds the average maximum rooting depth of 15.0 ± 5.4 m for tropical grassland or tropical savannah, respectively, which is the biome with the deepest-reaching roots (Canadell et al. 1996). Almost all phreatophytic tree and shrub species found in the survey exhibited maximum rooting depths of more than 2 m, which, on a global scale, is typical of these plant growth forms in water-limited environments (Schenk and Jackson 2002).

Specimens of phreatophytic species (*Alhagi sparsifolia*, *Populus euphratica*, *Tamarix ramosissima*) have been found growing at a distance of more than 15 m

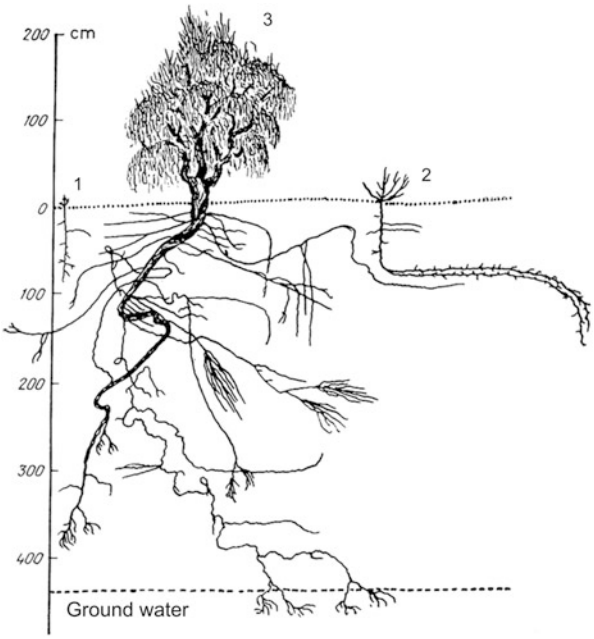

Fig. 2 *Haloxylon aphyllum* with root system. (1) 1-year-old seedling; (2) 2-year-old sapling; (3) ca 15- to 20-year-old tree (from Walter and Breckle 1994, modified). Specimens of this species only form large plants and dense vegetation when their roots have reached the groundwater

to the water table and obviously completely rely on groundwater. This can be concluded from circumstantial evidence as the annual precipitation is less than 50 mm, the soil water contents above the groundwater's capillary fringe are lower than 5 % and soil water potentials lower than −10 MPa, and the plants transpire continuously during the vegetation period (Gries et al. 2003; Thomas et al. 2006a; Bruelheide et al. 2010). In this case, the question arises how these plants became established. Obviously, this is only possible at sites and in periods where the upper soil has been thoroughly wetted during flooding by a nearby river, and the distance to the groundwater is small enough to be bridged by rapid vertical root growth. At least some of the phreatophytic species exhibit a vertical root growth of more than 2 m during a vegetation period when there is enough water available in the upper soil layers (see above). This is sufficient to tap shallow groundwater. Thereafter, the distance to the groundwater may become larger by lowering of the water table (due to groundwater use by the human population or by natural shifts in the course of a river) or by sand accumulation. Some phreatophytic species such as *Tamarix* spp. but also *Nitraria* species and others are particularly effective in building cone-shaped dunes, so-called *nebkhas*, by accumulating sand (e.g. Qong et al. 2002). Species whose vertical root growth cannot keep pace with the increasing distance to the water table disappear, and the average number of species decreases. Remaining species that are tolerant to increased salt concentrations of the soil have an advantage as the salt is not leached any more from the soil by flooding or inundations (Thevs et al. 2008b). Due to continuous sand accumulation, the canopy of phreatophytic species whose shoot growth can keep pace with the accumulating sand

eventually is positioned at a distance of much more than 10 m above the water table without losing contact to the groundwater. This process explains the occurrence of phreatophytic vegetation at sites with a long distance to the water table (cf. Fig. 1). At such sites, however, rejuvenation of the stands can be hampered because the soil surface is not flooded any more by water courses; in *Populus euphratica*, more or less regular flooding seems to be a prerequisite of regeneration by root suckers. Thus, those stands eventually will die off (Runge 2004). Accordingly, extensive dead stands of *P. euphratica* can be seen along the Central-Asian Tarim River at locations that are now a long way away from the river due to shifts of the river course, or due to excessive use of groundwater for agriculture (cf. Feng et al. 2005).

Vegetative regeneration plays an important role in phreatophytes. Beginning at an age of 15–20 years, *Populus euphratica* forms suckers from roots that stretch almost horizontally within the uppermost 0.6 m of the soil; the suckers emerge at a distance of up to 40 m from the parent trees (Wiehle et al. 2009). Vigorous sprouting from trunks and stems as well as suckering from underground organs has also been observed in North-American *Populus* species after fire, in particular, in *P. angustifolia, P. balsamifera* ssp. *balsamifera*, and *P. balsamifera* ssp. *trichocarpa*, but also in *P. deltoides* (Gom and Rood 1999). According to own observations, extensive underground runners are also formed by the Central-Asian shrub species *Calligonum caput-medusae* and *Tamarix ramosissima* as well as by the sub-shrub *Alhagi sparsifolia*. The strong vegetative regeneration results in the formation of large clones that can cover areas of considerable size. Maximum clone sizes detected at the southern fringe of the Central-Asian Taklamakan Desert were 6.1 ha in *Alhagi sparsifolia* and 121 ha in *P. euphratica* (Vonlanthen et al. 2010b). In most probability, these clones also have an old age. The maximum size of *Tamarix ramosissima* clones was distinctly smaller (38 m^2; Vonlanthen et al. 2010b). This might be attributed to the growth habit of that species, which is capable of forming dome-shaped dunes ("cones") with a typical height of 3–15 m. This is achieved through sand and litter accumulation, and through maintaining rates of height increment that allow for keeping pace with the sand accumulation. On the basis of radiocarbon dating, an age of approximately 4,200 years has been determined for older *Tamarix* cones (cf. Qong et al. 2002).

2.2 Water and Nutrient Relations of Phreatophytes

2.2.1 Water Transport

Although phreatophytes have access to the water-saturated zone of the soil, the supply of the plants' canopy with sufficient amounts of water per unit time can be challenged by high rates of water loss caused by high transpirational demands due to a large water vapour pressure deficit of the air (VPD). Whilst daily VPD maxima of more than 3 kPa are uncommon in temperate regions and only occur during very

warm and dry periods, this value can be exceeded on more than 80 % of the growing season's days in phreatophyte habitats of hyper-arid deserts, and peak values of more than 5.5 kPa can be reached (Thomas et al. 2008). Thus, for effectively transporting water from the saturated zone of the soil to the canopy of the plant, the tap root and the shoot should have a hydraulic conductance that is efficient enough to provide the canopy with sufficient amounts of water, and the plant should regulate its water loss so tightly that desiccation is avoided.

The hydraulic conductivity of plants can be determined as the amount of water transported along a given distance per unit area, water potential gradient, and time (1) by direct measurements of the flow of water or an aqueous solution per unit cross-sectional area through excised sections of the roots or the shoot; this method has been described by Huber (1956) (cited after Lösch 2001) and, after refinement by Sperry et al. (1988), has been successfully used with modifications in several studies during the past decades; (2) by determining the leaf area-related transpiration—quantified by measuring the xylem sap flow or micrometeorological variables—along the water potential gradient from the soil to the leaves; or (3) by calculating the rate of xylem sap flow per unit root or shoot cross section and unit water potential gradient using the Hagen–Poiseuille equation on the basis of the xylem conduit dimensions (e.g. Tyree and Zimmermann 2002). Table 2 provides data of the leaf area-related and the calculated hydraulic conductivity of some phreatophytic species.

The values of leaf area-related hydraulic conductivity on the flow path from the soil to the leaves (k_{SL}) determined in woody phreatophytes are distinctly higher than those obtained from conifers growing under temperate or boreal climate (*Larix* sp., *Picea abies*, *Pinus pinaster*; <0.5 $mmol_{H2O}$ m^{-2} s^{-1} MPa^{-1}) as well as those measured in ring-porous *Quercus* species (*Q. marilandica*, *Q. rubra*; ≤ 2.1 $mmol_{H2O}$ m^{-2} s^{-1} MPa^{-1}) (cf. Wullschleger et al. 1998). Markedly higher values than those found in phreatophytes (>9 $mmol_{H2O}$ m^{-2} s^{-1} MPa^{-1}) were only computed for tree species growing in moist environments such as lowland tropical moist forests (e.g. in *Cecropia insignis*, a rapidly growing gap pioneer; and in *Miconia argentea*, a deep-rooting tree with poor stomatal control of transpiration in the wet season; Meinzer et al. 1995), and in desert sub-shrubs under ample water supply (approximately 10–18 $mmol_{H2O}$ m^{-2} s^{-1} MPa^{-1}; cf. Mencuccini 2003). The extraordinarily high k_{SL} value of *Alhagi sparsifolia* specimens (23.7 $mmol_{H2O}$ m^{-2} s^{-1} MPa^{-1}), which grow at the southern fringe of the hyper-arid Taklamakan Desert in Northwestern China, was measured during the period of rapid above-ground biomass formation in May. When maximum biomass had been reached in June, k_{SL} decreased to less than 2 $mmol_{H2O}$ m^{-2} s^{-1} MPa^{-1}. In most probability, the high k_{SL} value of *A. sparsifolia* was a causal factor of high growth rates during this period, and of the short period of time in which above-ground biomass formation was completed (56 days, as opposed to 77 and 79 days in the co-occurring *Populus euphratica* and *Tamarix ramosissima*; Thomas et al. 2008). The high hydraulic conductivity also is a prerequisite for sustaining growth at the large distance to the groundwater (approximately 16 m in *A. sparsifolia*).

Table 2 Hydraulic conductance and vulnerability to cavitation of several phreatophytic species

Species	Growth form	Distance to ground water (m)	k_{SL} (mmol m^{-2} s^{-1} MPa^{-1})	k_{SA} (kmol m^{-1} s^{-1} MPa^{-1})	Ψ_{PLC50} (MPa)
Alhagi sparsifolia	Sub-shrub	ca 16	23.7[a]	3.8[b]	n.d.
Populus euphratica	Tree	3.6	2.6[a]	1.5[b]	−0.7[d]
Populus fremontii	Tree	2.0	4.5[c]	n.d.	−1.5 to −1.6[e]
Salix gooddingii	Tree	2.0–3.6	5.9[c]	n.d.	−1.4 to −1.5[e]
Tamarix ramosissima	Shrub	5.7	3.7[a]	1.2[b]	−4.5[f]
Tessaria sericea	Shrub	3.6	3.6[c]	n.d.	n.d.

Maximum values of leaf area-related hydraulic conductivity on the flow path from the soil to the leaves (k_{SL}), theoretical hydraulic conductivity along a shoot section of unit length calculated using the Hagen–Poiseuille equation on the basis of the xylem conduit dimensions and related to unit shoot cross-sectional area (k_{SA}), and xylem water potential at 50% loss of maximum hydraulic conductance (Ψ_{PLC50}). The distance to groundwater refers to the k_{SL} and k_{SA} values

n.d. not determined

References: [a]Thomas et al. (2008), [b]Rzepecki et al. (2011), [c]Busch and Smith (1993), [d]Hukin et al. (2005), [e]Pockman et al. (1995), Horton et al. (2001b), [f]Pockman and Sperry (2000)

2.2.2 Xylem Anatomy and Vulnerability by Cavitation

A structural precondition of a high hydraulic conductivity is the presence of xylem conduits with large diameters. In accordance with its high k_{SL} value, *Alhagi sparsifolia* had a substantial fraction of large conduits with diameters of up to 240 µm (Rzepecki et al. 2011). Conduits of a similar size can also be found in the wood of ring-porous tree species (in which the largest conduits of all woody plants can be found) of Mediterranean or temperate regions (e.g. *Castanea sativa*, García-González and Fonti 2006; *Quercus* spp., Thomas et al. 2006b; Fonti et al. 2009; *Ulmus minor*, Solla and Gil 2002). These large conduits contribute to the high theoretical hydraulic conductivity per unit shoot cross section calculated using the Hagen–Poiseuille equation (k_{SA}) (Table 2). Maximum conduit diameters are distinctly lower in the phreatophytic shrubs *Prosopis alpataco* (152 µm; Villagra and Junent 1997) and *Tamarix ramosissima* (nearly 130 µm) as well as in the tree species *Populus euphratica* (110 µm) (Rzepecki et al. 2011). Nevertheless, the k_{SA} values of the latter two phreatophytic species are still higher than those determined in the temperate ring-porous *Quercus robur* (0.6 $kmol_{H2O}$ m^{-1} s^{-1} MPa^{-1}; Steppe and Lemeur 2007), and distinctly higher than in mesic diffuse-porous tree species (*Fagus sylvatica*, 0.3 $kmol_{H2O}$ m^{-1} s^{-1} MPa^{-1}, Steppe and Lemeur 2007; and *Betula pendula*, 0.1–0.3 $kmol_{H2O}$ m^{-1} s^{-1} MPa^{-1}, Sellin et al. 2008).

Large conduits that facilitate a high hydraulic conductance and, thus, high rates of water transport might also be more prone to drought-induced cavitation, i.e. the rupture of the water column in the xylem conduits due to air suction through relatively large pores of the conduit's pit membrane (Hacke and Sperry 2001). Xylem vessels with a larger diameter and a larger surface area exhibit a larger pit membrane area per vessel and, therefore, a higher susceptibility to cavitation (Hacke et al. 2006). This might be explained by a higher probability of the occurrence of large pores, or by a parallel increase in pit area and maximum pore size, in large pit membrane areas (Wheeler et al. 2005). Accordingly, recent studies (e.g. Taneda and Sperry 2008; Hacke et al. 2009; Hölttä et al. 2011) again found that larger conduits are more vulnerable to cavitation than smaller ones, and thus corroborated similar previous results (e.g. Lo Gullo and Salleo 1991; Lo Gullo et al. 1995).

A commonly used measure of the xylem's vulnerability to cavitation is the water potential at which the xylem of a root or shoot section has lost 50 % of its maximum hydraulic conductance (Ψ_{PLC50}) — the lower (more negative) the Ψ_{PLC50}, the lower is the vulnerability to cavitation. In phreatophytic *Populus* and *Salix* species, Ψ_{PLC50} values were not below −1.6 MPa (Table 2). Ψ_{PLC50} values that were somewhat more negative were determined in Western Australian *Banksia* species. "Obligate" phreatophytic *Banksia* species (*B. ilicifolia*, *B. littoralis*), which grew at a distance to the groundwater of less than 8 m, displayed less negative Ψ_{PLC50} values (−1.8 MPa) than the "facultative" phreatophytes *B. menziesii* (−2.0 MPa) and *B. attenuata* (−2.5 MPa) that could sustain a distance to the water table of more than 30 m (Canham et al. 2009). The authors explained their results with a higher phenotypic plasticity of the "facultative" phreatophytic species. Similarly, the

"facultative" phreatophytes *B. attenuata* and *B. menziesii* were less vulnerable to cavitation than the "obligate" phreatophyte *B. ilicifolia* (Froend and Drake 2006). Even in the "facultative" phreatophytic *Banksia* species, however, Ψ_{PLC50} is relatively high compared to respective values determined in ring-porous *Quercus* species growing under temperate climate (*Q. robur*, −2.7 MPa; *Q. petraea*, −3.3 MPa), or in the typical Mediterranean species *Q. ilex* (−6.0 MPa) (Tyree and Cochard 1996). Maherali et al. (2004), who found a significant correlation between Ψ_{PLC50} and hydraulic conductance across 167 angiospermous and coniferous species, calculated median Ψ_{PLC50} values for five vegetation types (tropical rain forest, tropical dry forest, temperate forest, desert and Mediterranean vegetation) in which the considered species grow. The median Ψ_{PLC50} values were equal to or lower than −2.4 MPa in all these vegetation types except for the tropical rain forest, where they exceeded −1.0 MPa. This demonstrates that the Ψ_{PLC50} values determined in phreatophytes really are relatively high, and are indicative of a relatively high vulnerability to cavitation. Such relationships were also found in a comparative study on desert shrubs growing in the North-American Great Basin, which demonstrated that phreatophytic species were more vulnerable to cavitation than co-occurring non-phreatophytes (Sperry and Hacke 2002).

2.2.3 Gas Exchange and Its Regulation

For preventing excessive water loss, anatomical and morphological features of the above-ground organs such as leaf reduction or xeromorphic leaves with well-developed cuticles as well as a sensitive regulation of the stomatal closure are most important adaptations to a high transpirational demand in plants growing in dry environments. However, in accordance with a high hydraulic conductance, phreatophytes often display high values of foliar conductance to water vapour (g_L). Maximum g_L values of approximately 250 $mmol_{H2O}\ m^{-2}\ s^{-1}$ have been found in *Prosopis flexuosa* woodland of the Argentine Monte Desert (Giordano et al. 2011), of 280 $mmol_{H2O}\ m^{-2}\ s^{-1}$ in the shrub *Sarcobatus vermiculatus* (Trent et al. 1997), of 600 $mmol_{H2O}\ m^{-2}\ s^{-1}$ in the shrub *Tamarix gallica* growing in the Great Plains (Mounsif et al. 2002), of 700 $mmol_{H2O}\ m^{-2}\ s^{-1}$ in the desert sub-shrub *Alhagi sparsifolia* during the period of rapid above-ground biomass formation (Thomas et al. 2006b), and of 800–900 $mmol_{H2O}\ m^{-2}\ s^{-1}$ in the tree species *Populus angustifolia* and in the shrubs *Salix exigua* and *S. monticola* growing at high elevations in arid woodland (Foster and Smith 1991). These values exceed the maximum g_L values that were found to be typical of desert shrubs and of woodland species growing in seasonally dry regions (approximately 180–240 $mmol_{H2O}\ m^{-2}\ s^{-1}$; Körner 1995). However, lower g_L values have also been obtained from riparian shrubs and trees (approximately 100, 140, and 150 $mmol_{H2O}\ m^{-2}\ s^{-1}$ in *Tamarix ramosissima*, *Populus euphratica*, and *P. fremontii*, respectively; Busch and Smith 1995; Gries et al. 2003); these values are more typical of deciduous shrub or tree species growing in arid regions. In contrast, maximum values of light-saturated

net photosynthesis (A_{max}) measured in phreatophytes (*Allenrolfea occidentalis*, *Sarcobatus vermiculatus*, Trent et al. 1997; *Chilopsis linearis*, *Prosopis glandulosa*, de Soyza et al. 2004; *Populus angustifolia*, Letts et al. 2008; *Tamarix gallica*, Mounsif et al. 2002; *T. ramosissima*, Xu et al. 2007) were within the range of values that are considered typical of shrubs or trees growing in (semi-)arid steppe and woodland outside the tropics (i.e. $\leq$20.5 $\mu mol_{CO2}\ m^{-2}\ s^{-1}$; cf. Woodward and Smith 1995). However, relatively high A_{max} was determined in trees of *Populus fremontii* and *Salix gooddingii* (up to 30 $\mu mol_{CO2}\ m^{-2}\ s^{-1}$), and surprisingly high values, which even exceeded 60 $\mu mol_{CO2}\ m^{-2}\ s^{-1}$, were measured in specimens of *Tamarix chinensis* that grew at relatively short distances to the groundwater (<3 m; Horton et al. 2001a). Altogether, when compared to transpiration rates, these findings are indicative of low water use efficiency in most phreatophytic species.

Due to the high transpirational demand that is widespread in (semi-)arid habitats, even phreatophytes with permanent access to the water table have to regulate transpiration, especially when the transpiration rates would become so high that the hydraulic conductance is not sufficient to supply the canopy with adequate amounts of water. Comparative studies have shown that the factors that regulate the foliar conductance to water vapour (g_L) are quite different among phreatophytic species. In the "facultative" phreatophytes *Prosopis glandulosa* (Nilsen et al. 1983) and *Retama sphaerocarpa* (Domingo et al. 2003) as well as in the "obligate" phreatophyte *Populus euphratica* (Thomas et al. 2008), foliar conductance is highly sensitive to an increase in VPD or in the leaf-to-air difference in the partial pressure of water vapour (Δw), respectively. In contrast, VPD explained only a small fraction (<29 %) of the variation in g_L of *Populus fremontii*, *Salix gooddingii*, and *Tamarix chinensis* (Horton et al. 2001a). Moreover, the stomata of the two Salicacean species seem to operate with only narrow safety margins above cavitation thresholds: complete xylem cavitation was observed when the leaf water potential fell only slightly below the Ψ_{PLC50} value of -1.5 to -1.6 MPa (Pockman et al. 1995). This might be related to the fact that these species principally occur at low distances to the groundwater ($\leq$5 m; cf. Table 1), whereas the above-ground parts of *P. euphratica* can grow at a groundwater distance of 23 m (Gries et al. 2003); under these conditions, a high sensitivity of g_L to a decrease in VPD is necessary to avoid too large losses of water and too negative water potentials, which would result in xylem cavitation. In contrast, the leaf water potential contributed substantially to explaining the variation of g_L in *Tamarix ramosissima* (Thomas et al. 2008). This species, which can thrive at a groundwater distance of 24 m (Gries et al. 2003), also displayed a much lower Ψ_{PLC50} value (Table 2) and a wider safety margin above the threshold of complete cavitation (Pockman and Sperry 2000); this also renders the plant less susceptible to high VPD values. Low Ψ values of the halophytic *Tamarix* species are also caused by substantial salt accumulation in their leaves (Di Tomaso 1998; Arndt et al. 2004). In *Alhagi sparsifolia*, a species that can grow at a distance of up to 20 m to the water table (Vonlanthen et al. 2010b), g_L was most closely related to k_{SL} (Thomas et al. 2008). In most probability, this is related to the high growth rate and the short period that is needed to complete the

production of above-ground biomass (see Sect. 2.2.1). Altogether, in phreatophytes, the VPD, the distance to the water-saturated soil layers, the hydraulic conductance of the xylem, the capability of accumulating osmotically active compounds in the leaves, and the rates of biomass formation all seem to play important roles in the regulation of g_L, but in species-specific extents and combinations.

Cooling the leaves by high rates of transpiration is a peculiar adaptation of broad-leaved phreatophytes to a hot climate. Evidence for this mechanism has been provided by Lange (1959) by investigating leaves of *Citrullus colocynthis*, a hygromorphic species that grows in the humid soils of gullies at the fringes of the hot Saharo-Sindian deserts (Walter and Breckle 2004). A more recent experiment (Althawadi and Grace 1986) confirmed the results of Lange's study by showing that the leaf temperature of *C. colocynthis* could be kept up to 7 °C below air temperature by transpiration rates of up to 30 $mmol_{H2O}\ m^{-2}\ s^{-1}$, whereas the temperature of non-transpiring, dry model leaves distinctly exceeded the air temperature. High transpiration rates of up to 14 $mmol_{H2O}\ m^{-2}\ s^{-1}$, which might even exceed transpiration rates of well-watered mesophytes, have also been measured in other broad-leaved desert plants (Smith 1978). It seems that this intense transpirational cooling in desert environments is only possible when the plants have access to groundwater. In most probability, transpirational cooling is a prerequisite of survival of broad-leaved phreatophytes growing in warm deserts (Gibson 1996).

3 The Role of Phreatophytes for Ecosystem Functioning and Structure

In deserts, water use through transpiration by phreatophytes generally is the predominant process of water discharge (Ostrovsky 2007). For determining the water use of plant stands, most studies employ the Bowen ratio energy balance approach on the basis of meteorological data, or the leaf area-related plant transpiration obtained through measurements of the xylem sap flow in representative shoots. For both methods, the leaf area index (LAI, i.e. the sum of one-sided leaf areas per unit soil surface area) is needed. In accordance with the high hydraulic and stomatal conductance that generally can be found in phreatophytes (see Sects. 2.2.1 and 2.2.3), and owing to the high VPD that often prevails in the environments in which phreatophytes grow, the soil surface area-related water use of phreatophytic vegetation can be considerable. In C_3 trees and shrubs growing in the transition zone between oasis and desert at the southern fringe of the hyper-arid Taklamakan Desert in Central Asia as well as in arid *Banksia* woodland of Southwestern Australia, water use that approaches or even exceeds 400 mm a^{-1} has been determined, and an even higher value has been calculated for an alkali meadow with a small distance to the water table in the Great Basin of Southwestern North America (Table 3). At the southern fringe of the Taklamakan Desert, the annual water use by the C_3 vegetation contributes more than 90 % to the total evapotranspiration as the annual

Table 3 Maximum values of annual water use of phreatophytic vegetation, and distance to the groundwater and annual precipitation at the respective study sites

Species	Growth form	Ecosystem	Distance to groundwater (m)	Precipitation (mm a^{-1})	Water use (mm a^{-1})	Reference
Alhagi sparsifolia	Sub-shrub	Transition zone oasis—hyper-arid desert	ca 16	33	391[a]	Thomas (2004)
Banksia attenuata, *B. menziesii*	Tree	*Banksia* woodland on sand dunes	6–7	635	387[b]	Dodd and Bell (1993a)
Calligonum caput-medusae (C_4 species)	Shrub	Transition zone oasis—hyper-arid desert; plantation	ca 12	33	183[a]	Thomas (2004)
Sporobolus airoides/ *Distichlis spicata*	Grass	Alkali meadow	1.3–2.1	130	520[b]	Steinwand et al. (2006)
Populus euphratica	Tree	Transition zone oasis—hyper-arid desert	3.6	33	465[c]	Thomas (2004)
Quercus douglasii	Tree	Oak savanna	ca 8	600	210[a]	Miller et al. (2010)
Sarcobatus vermiculatus	Shrub	Arid shrubland	1.8	n.d.	308[b]	Nichols (1994)
Tamarix ramosissima	Shrub	Transition zone oasis—hyper-arid desert	5.7	33	441[a]	Thomas (2004)

n.d. not determined

Method of water use determination: [a]xylem sap flow, [b]energy balance (Bowen ratio), [c]foliar transpiration under conditions of close coupling between the conditions at the leaf surface and in the surrounding atmosphere, cf. Thomas et al. (2006a)

precipitation in this region is as low as 33 mm (Thomas et al. 2000, 2006a). In this environment, groundwater in soil depths of less than approximately 30 m is fed by rivers that are supplied with water by the melting of snow and glaciers in the adjacent mountains. But also in a Californian oak (*Quercus douglasii*) savannah, approximately 80 % of the total evapotranspiration during the summer months (June through August) came from transpired groundwater (Miller et al. 2010). The high values of water use at the fringe of the Taklamakan Desert distinctly exceed rates that are considered typical of desert vegetation (4–150 mm a^{-1}), and are within the range of water use determined for deciduous forests of temperate humid zones (300–600 mm a^{-1}; Larcher 2003). The high water use by phreatophytes, in particular by invasive *Tamarix* species in southwestern North America, is also of concern to water managers (Sala et al. 1996; see also Sect. 4.3).

In accordance with the high water use, the productivity of above-ground phytomass can also be high. At the southern fringe of the Taklamakan Desert, an annual production of 6.1 and 7.1 Mg_{DM} ha^{-1} has been determined for *Populus euphratica* and *Tamarix ramosissima*, respectively; in plantations of the C_4 shrub *Calligonum caput-medusae*, the annual above-ground productivity even was as high as 11.3 Mg_{DM} ha^{-1} (Gries et al. 2005). For *Phragmites australis*, maximum productivity of 6.0 Mg_{DM} ha^{-1} a^{-1} has been determined (Zerbe and Thevs 2011). In accordance with the high values of water use, the annual above-ground phytomass production also clearly exceeds the maximum net primary productivity of typical desert scrub vegetation (2.5 Mg_{DM} ha^{-1} a^{-1}), and is already within the range of the productivity of warm temperate mixed forests (6–25 Mg_{DM} ha^{-1} a^{-1}; Lieth 1975). Despite the high values of above-ground productivity, the water use efficiency of production (WUE_P, above-ground biomass produced per unit water transpired) of phreatophytes is low due to their ample use of water. When maximum values of above-ground phytomass production were related to the maximum values of water use, maximum WUE_P was as low as 1.8 g_{DM} $kg_{H2O}{}^{-1}$ (May through July in *Alhagi sparsifolia*) in phreatophytes growing at the southern fringe of the Taklamakan Desert (Thomas 2004). Thus, WUE_P of phreatophytes seems to be markedly lower than WUE_P of non-phreatophytic species, which exhibit values of 3–5 g_{DM} $kg_{H2O}{}^{-1}$ in broad-leaved trees of temperate regions and in C_4 plants, and 3–6 g_{DM} $kg_{H2O}{}^{-1}$ in sclerophyllous shrubs (Larcher 2003). Nevertheless, distinct interspecific differences in the water use efficiency can be found among phreatophytes. Ratios of stable carbon isotopes provide evidence that the water use efficiency is higher in *Tamarix ramosissima* when compared to the co-occurring *Populus fremontii*, *Salix gooddingii*, and *Tessaria sericea* in the Colorado River floodplain (Busch and Smith 1995), or to the co-occurring *Alhagi sparsifolia* and *Populus euphratica* in the Central-Asian Taklamakan Desert (Thomas et al. 2006a). In most probability, this is at least partly related to the halophytic character of *T. ramosissima* (Busch and Smith 1993).

Together with water, considerable amounts of nutrients can be taken up by phreatophytes. On the arid Colorado Plateau, a vegetation cover of 25 %, composed of the shrubby phreatophytes *Atriplex canescens* and *Sarcobatus vermiculatus*,

would be capable of taking up 100 kg nitrogen per hectare and year from the groundwater according to calculations (McKeon et al. 2006).

Some studies deal with the role of phreatophytes in structuring the ecosystems in which they predominate. Klopatek and Stock (1994) provide an example of a dune-dwelling phreatophytic shrub (*Acanthosicyos horridus*), endemic to the Namib Desert, which creates micro-ecosystems within the dunes. These micro-ecosystems provide shelter, food, and water (owing to large water contents in many plant parts) for several species at different trophic levels. Walter and Breckle (1994) cite studies on *Haloxylon aphyllum* stands that describe mosaic structures of vegetation under the tree canopy layer, which are characterised by an increased occurrence of hygrophilic, nitrophilic, and halophilic herbs due to alterations in the microclimate and in the soil conditions compared to the conditions outside the *Haloxylon* stands. The composition of the herbal plant community changes along subsequent stages of sand accumulation, sand fixation, and ageing of the *Haloxylon* trees. In contrast, thickets of invasive *Tamarix* species can hamper the establishment of other species and have a large impact on the water relations of the ecosystem. This subject will be covered in a special Sect. 4.3 of the following chapter.

4 Phreatophytes and Land Management

4.1 Use of Phreatophytes for Management and Monitoring Purposes

In arid and semi-arid regions, soil erosion by wind or water and sand encroachment to agricultural areas and settlements belong to the principal factors that result in land degradation and desertification (Geist 2005). Phreatophytes play an important role in preventing soil erosion and in the restoration of vegetation due to their capability of fixing sand. This has been shown, *inter alia*, for *Prosopis glandulosa* in the Mojave Desert of California (Laity 2003), and for *Alhagi sparsifolia*, *Calligonum caput-medusae*, *Karelinia caspia*, *Populus euphratica*, and *Tamarix ramosissima* in the Central-Asian Taklamakan Desert (e.g. Xia et al. 1993; Ci and Yang 2010).

Due to the close contact of phreatophytes with groundwater, phreatophytic vegetation can be used to map regional groundwater systems (Batelaan et al. 2003). For the same reason, phreatophytes can be employed to monitor the quality of the groundwater. In *Prosopis velutina* Woot. (=*P. articulata* S. Watson) growing in the Sonoran Desert of Southern California, electrical conductivity and peroxidase activity of leaf extracts correlated with the salinity of the groundwater (García-Carreño et al. 1992). The concentrations of boron, bromine, sodium, and strontium in leaves of *Populus deltoides* reflected the respective concentrations in contaminated groundwater (Erdman and Christenson 2000). In phreatophytic shrubs (*Atriplex canescens*, *Sarcobatus vermiculatus*) that grew on a nitrate-contaminated

aquifer, the foliar nitrate concentration exhibited a fivefold increase (McKeon et al. 2006). According to calculations of the authors, restored shrub vegetation that also is protected from grazing could significantly contribute to a decontamination of the groundwater.

4.2 Effects of Changes in the Groundwater Level

In many regions, phreatophytic vegetation suffers from natural but also from widespread anthropogenic alterations of the groundwater level. This has been found for different growth forms of phreatophytes in North America as well as in Central Asia. Lowering of the water table caused an increase in dieback of *Populus fremontii* and *Salix gooddingii* growing in floodplains of West-Central Arizona (Shafroth et al. 2000; Horton et al. 2001a), and of *Prosopis glandulosa* in the Californian Mojave Desert (Laity 2003). The dieback of entire *Populus euphratica* forests along the lower reaches of the Tarim River in Xinjiang, Northwestern China, was ultimately caused by an overuse of the river's water resources at its upper and middle reaches (Feng et al. 2005). The survival of young *Chrysothamnus nauseosus* shrubs in a Californian sand dune ecosystem located in the rain shadow of the Sierra Nevada was found to be strongly related to the depth of the water table (Toft and Fraizer 2003). In a Californian grassland, a decrease of the water table below the rooting zone resulted in a severe decline in the cover of phreatophytic grasses (Pritchett and Manning 2012). A decline in the water table can also result in a change of the vegetation's species composition, e.g. from grass to shrub dominance (Pritchett and Manning 2012) or, more generally, in an increase in the abundance of drought-tolerant species at the expense of species that prefer wetter sites (Froend and Sommer 2010). In most probability, a decrease in the vegetation cover and changes in the vegetation composition might also be related to the loss of the groundwater's buffering effect: a decline of the water table below the rooting zone makes the plants more dependent on precipitation and renders them more susceptible to changes in the precipitation's annual amount and distribution (Elmore et al. 2006).

In addition to the distance to the water table itself, the extent of the decline in water table affects the mortality of phreatophytes. In the floodplains of West-Central Arizona, the mortality of *Populus fremontii* and *Salix gooddingii* saplings was higher at a site with a smaller distance to the groundwater, but with a more severe interannual decline of the water table than at a site with a larger distance to the water table, but less change between years; in most probability, the differences in sapling mortality between the sites were due to the conditions under which the roots were formed (Shafroth et al. 2000).

Not only a decline in the groundwater level but also a rise in the water table can induce stress to phreatophytes: flooded roots can be damaged or killed under the developing hypoxic or anoxic conditions (Naumburg et al. 2005). For instance, the

phreatophytic tree species *Carya illinoensis*, which is endemic to the Mississippi River valley, turned out to be sensitive to poor drainage (Sparks 2005).

4.3 *The Case of Invasive* Tamarix *Species in Southwestern North America*

The genus *Tamarix*, which comprises 67 species (Baum 1978), is native to Asia, the Mediterranean region including North Africa, and Southern Europe. Since 1800, eight to twelve *Tamarix* ("saltcedar") species have been introduced to North America for the provision of shade and the control of erosion (Gaskin and Schaal 2002). For these purposes, *Tamarix* species were highly esteemed due to their high salt and drought tolerance (Chew 2009). It was just these features that contributed to making them invasive: by the end of the 1980s, an area estimated to be as large as 600,000 ha has been invaded by *Tamarix* species (Di Tomaso 1998). Together with water shortages in (semi-)arid regions of the southern and southwestern USA, the habit of *Tamarix* species to use relatively large quantities of water changed their public perception. They were now widely regarded as "foreign monsters" (Chew 2009), and large efforts were made to control them, including application of herbicides from aircrafts (e.g. Duncan and McDaniel 1998).

The species that now are most widespread in North America are *T. chinensis* and *T. ramosissima*, which have a similar morphology. However, genetic analyses demonstrated that a hybrid between these two species is the most common plant of the invasion and occurs from Oklahoma to Washington and California (Gaskin and Schaal 2002). Thus, several of the *Tamarix* plants that have been attributed to one of the two parent species in the numerous studies on invasive *Tamarix* species in North America (e.g. Busch et al. 1992; Devitt et al. 1997; Horton et al. 2001a) might rather have been hybrids, but to avoid confusion, I have retained the species names as were given in the original sources.

In southwestern North America, invasion by *Tamarix* species was particularly intense (Di Tomaso 1998). A combination of several morphological and physiological traits can explain this invasion success. *Tamarix* stands can exhibit extremely high rates of evapotranspiration, in particular in dense stands at short distances to the groundwater (Smith et al. 1998). This is due to the maintenance of a large leaf area (high LAI) even under drier soil conditions and at a high transpirational demand (Sala et al. 1996; Horton et al. 2001b). In thickets of *T. chinensis*, evapotranspiration did not decrease but even increase upon a decline of the water table (Cleverly et al. 2006). Moreover, substantial night-time evapotranspiration can occur, which, in *T. chinensis*, can contribute as much as 37 % to daily total evapotranspiration when enough water is available (Moore et al. 2008). *Tamarix ramosissima* is also capable of rapidly switching from the use of groundwater to water uptake from non-saturated soil layers upon a decline in the water table (Nippert et al. 2010). Due to its intense water use, *Tamarix* can lower the water

table by itself (Di Tomaso 1998) and, thus, induce a more intense drought stress to co-occurring species. After application of drought stress by suspension of irrigation for 4 weeks, xylem sap flow, stomatal conductance, and leaf water potential of *T. ramosissima* rapidly recovered to pre-drought values upon rewatering (Devitt et al. 1997). Due to these characteristics, *Tamarix* is less susceptible to adverse effects induced by a decline of the water table than co-occurring native phreatophytes such as *Pluchea sericea*, *Populus fremontii*, *Prosopis pubescens*, *Salix exigua*, and *Salix gooddingii* (Cleverly et al. 1997; Horton et al. 2001a, 2003; Stromberg et al. 2010). Accordingly, in contrast to *Populus euphratica*, the stem diameter increment of *T. ramosissima* in its native area did not decrease along an increase of the groundwater distance from 5 to 24 m (Gries et al. 2003). Increasing drought stress through a decrease in the availability of soil moisture or to an increase in the transpirational demand may thus result in a change in the composition of the plant community towards more stress-tolerant species such as *Tamarix* taxa, and also to a reduction in animal diversity (Smith et al. 1998). In riparian vegetation, *Tamarix* recovers more quickly from wildfire than the co-occurring *Populus* and *Salix* species, and can thus gain even greater dominance in the plant community (Smith et al. 1998). Possibly, *Tamarix* can also hamper the germination of co-occurring (and potentially competing) plant species by salinization of the soil due to salt excretion or to shedding its leaves that have a high salt concentration (Smith et al. 1998; Glenn et al. 2012; Imada et al. 2012). In addition, its small seeds can easily be distributed over large distances by wind or water. However, they are viable during a short period of time only and have to get into contact to water within a few weeks after dispersal (Di Tomaso 1998). Nevertheless, it is the anthropogenic change of hydrological regimes that still can be considered the principal factor of the spread of invasive *Tamarix* species into riparian ecosystems of the southwestern USA, and of subsequent alterations in community composition (e.g. Stromberg et al. 2007).

5 Conclusions

Despite distinct interspecific differences, many phreatophytes share the following anatomical and physiological traits: (1) a significant fraction of xylem conduits with large diameters; (2) high hydraulic conductance of the shoot, and along the flow path from the soil to the leaves; (3) relatively high vulnerability of the shoot to xylem embolism; (4) high foliar conductance to water vapour (which is species-specifically regulated in response to VPD, hydraulic conductance, or leaf water potential); (5) high rates of biomass production; and (6) low water use efficiency. On the basis of these shared structural and functional traits, one can suggest to regard phreatophytes as a hydro-ecological plant type, and to put them in a line with hydrophytes, hygrophytes, and xerophytes, which also are characterised by distinct anatomical, morphological, and physiological features within this context. However, overlapping may occur especially between phreatophytes and xerophytes as

several phreatophytes such as *Haloxylon* and *Tamarix* species do exhibit xeromorphic characters (sensu Metcalfe 1983), while others such as *Populus* and *Salix* species do not. As the fraction of the water demand that is covered by water uptake from the saturated zone of the soil seems to be determined by the prevailing environmental conditions at the sites of the phreatophytes' occurrence rather than by inherent traits of the plants, it is suggested to abandon the conventional terms "obligate" in favour of "permanent", and "facultative" in favour of "temporary", in the characterisation of phreatophytes.

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Some Like It Hot: Are Desert Plants Indifferent to Climate Change?

Katja Tielbörger and Roberto Salguero-Gómez

Contents

Abstract Deserts rank at the forefront of vulnerability to global change because their biota is expected to encounter large climatic changes while apparently existing at biological limits. We review the available evidence for climate change effects on arid lands, and specifically on vegetation because as primary producers, plants are main providers of ecosystem services. We summarize field experiments and correlative evidence from spatial and temporal climatic gradients. Surprisingly, only few climate manipulation experiments have been conducted in semideserts, none in arid regions, and almost none in cold drylands. We argue that correlative approaches do not yield the necessary knowledge to understand and thus mitigate potential changes due to their oversight of long-term evolutionary processes. Nonetheless,

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the limited mechanistic evidence suggests a surprisingly high resilience of desert vegetation to changes in precipitation and CO_2. We suggest this resilience is due to specific adaptations that have evolved in response to stressful and highly variable climatic conditions.

1 Introduction

Anticipating the ecological consequences of ongoing climate change is of utmost importance in the research agenda of ecologists, since this knowledge is a prerequisite for guidance for mitigation and conservation that society requires from us (Walther et al. 2002; Thuiller 2007). A keystone to this aim is to forecast future species distributions and to identify ecosystems that are especially vulnerable to projected climatic scenarios (Sala et al. 2000; Thomas et al. 2004). Plants are of particular interest in the exploration of the effects of global change because, as primary producers, they represent the basic resources for all other consumers, humans included. Predictions about the response of vegetation to climate change are especially pressing for ecosystems whose functioning is mainly driven by temperature and/or precipitation. Such ecosystems should be particularly vulnerable to climate change (Tompkins and Adger 2004). Deserts represent the best example for highly vulnerable ecosystems because warming may drive plant species to their physiological limits, and because a decrease in precipitation will aggravate such effects. Indeed, global assessments have ranked deserts and semideserts at the forefront of vulnerability to global change (Sala et al. 2000; IPCC 2007a).

Deserts (extremely arid and arid subtropical climates sensu Meigs 1953; see Fig. 1) constitute the most extensive terrestrial biome (Millennium Ecosystem Assessment 2005) and, in spite of some historical misconceptions about their value (Maestre et al. 2012), they represent a rich hotspot of biodiversity of invaluable ecological, cultural, historical, and economic importance. In general, the over 5×10^7 ha (>40 % of the land surface; Reynolds et al. 2007) covered by drylands are home to an important range of living forms and ecological structures that include the origin of the CAM pathway (Lüttge 2004), Guinness records of life span such as the bristlecone pine (*Pinus longaeva*; Lanner and Connor 2001) or the creosote bush (*Larrea tridentata*; Vasek 1980), as well as records of resistance to high temperatures, salinity, and hydraulic failure (Halvorson and Patten 1974; Danin 1976).

In addition, drylands are sometimes densely populated and the highest human population growth rates are observed and projected for certain arid- to semiarid regions (e.g., the Middle East; Onigkeit and Twite 2011). Therefore, climate change effects on desert vegetation will be most likely aggravated by increasing human impact and unsustainable land use (Fedoroff et al. 2010). It is thus not surprising that understanding the sustainability of local resources (e.g., vegetation) and how climate change will affect their provisioning has been highlighted as top priority in the research agenda of the IPCC (2007a).

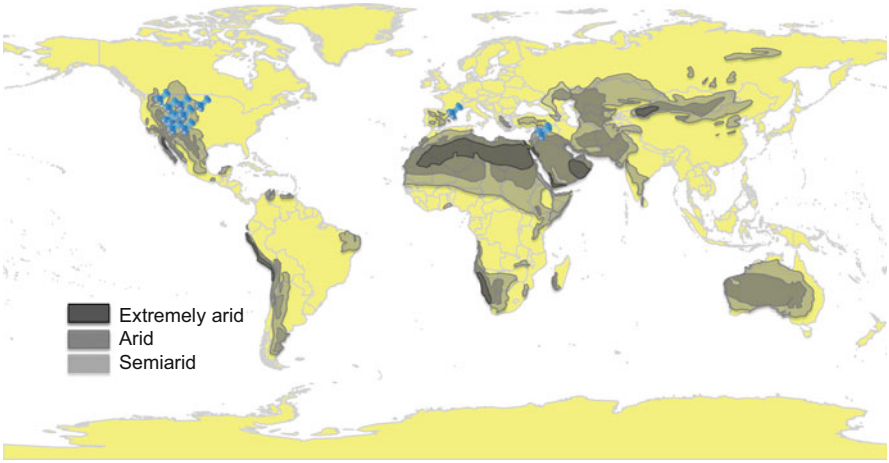

Fig. 1 Deserts of the world, classified into semiarid (*light gray*), arid (*gray*), and extremely arid (*dark gray*) regions (modified from Meigs 1953). The classification is based on Thornthwaite's (1948) moisture index which takes into account precipitation and evapotranspiration. It roughly corresponds to 250–500 mm annual precipitation for semiarid regions, less than 250 mm for arid regions, and more than 12 months of consecutive drought for extremely arid areas. Our review of the literature on the effects of climate change in deserts reveals an astonishing lack of experimental approaches in drylands in general, and these are limited to semiarid regions with a large geographic bias (see pins for locations of experimental studies reviewed here)

Arid lands are characterized by an overall low productivity because low precipitation levels do not, on average, match the evapotranspiration imposed by high solar radiation and temperatures (Thornthwaite 1948). However, a main feature of subtropical deserts is the inherently large and unpredictable intra- and interannual variation in precipitation (D'Odorico and Bhattachan 2012). Therefore, conditions for plant growth are not necessarily always unfavorable. Instead, resource demands are only temporarily fulfilled by pulses of precipitation of high intensity, rare occurrence, and even more challenging predictability. Such pulses may be intertwined with long periods of drought, a concept—the *pulse-reserve paradigm* (Noy-Meir 1973; Reynolds et al. 2004; Schwinning et al. 2004)—that is pivotal to the ecological and economic functions of arid regions.

Since dryland ecosystems are highly water limited already under current climatic conditions, robust ecological scenarios are urgently needed that quantify the vulnerability of these unique ecosystems to ongoing climate change. Two components of climate change, increase in temperature and shifts in precipitation pattern, are particularly crucial in the understanding of how the native flora and fauna of deserts will respond. Despite the projections from different scenarios, the general scientific agreement is that the increasing emission of greenhouse gases will result in warming of at least 3 °C and a doubling in the frequency of climatic extremes in drylands in the coming century (IPCC 2007b). Furthermore, these projections forecast increases in the degree of uncertainty of rainfall regimes,

ranging from a decrease of a third to an increase of a quarter of the annual precipitation (Bates et al. 2008). However, a large fraction of subtropical deserts will experience warming in combination with decreasing rainfall. In the meantime, other drivers of global change such as overgrazing, mismanagement practices in agriculture (Reynolds et al. 2001; Thornton et al. 2008), and man-induced desertification continuously increase the pressure on these ecosystems and may lead to irreversible degradation (Reynolds et al. 2007). For example, dramatic effects of climate change are expected in sub-Saharan Africa due to a combination of severe scenarios of change in precipitation and temperature, a high dependency of major sources of income (rainfed agriculture, livestock) on rainfall, and the severely limited adaptive capacity of the poor societies (Thornton et al. 2008).

The rate of change in these vulnerable ecosystems may far exceed the rate at which we are accumulating the necessary scientific evidence needed for managing them. In addition to the scientific duty that dryland researchers have towards providing solutions to sustaining the services provided by these ecosystems, other aspects also motivate dryland research. For one thing, deserts are excellent in vivo laboratories, allowing us to better understand the evolution of life under extreme conditions. Furthermore, obtaining a mechanistic understanding of how primary producers inhabiting systems "at the limit" will react to climate change will help us prepare for changes in regions that appear less vulnerable today but may soon be driven to their limits as well.

Here, we review direct and indirect evidence, also drawing from some of our own original research, for potential effects of climate change on desert vegetation. Our ultimate goal is to identify the most urgent research needs. We initially focused our review on regions that have been classified as arid and extremely arid (Fig. 1) and on vegetation characteristics that are relevant for vegetation structure, namely, plant density, species diversity, productivity, and species composition. However, due to the scarcity of relevant studies, and almost missing information from cold deserts except the Great Basin, we have also included semideserts (sensu Meigs 1953) into our literature survey.

2 Examining the Effects of Climate Change in Deserts

Three different approaches to studying climate change impacts on vegetation and plant community structure and function can be distinguished. (1) Spatial climatic gradients (e.g., altitude, longitude) are used as proxy for determining the response of ecosystems to future climate change (space-for-time substitution). Related to that approach, albeit with different assumptions, are bioclimatic envelope models that map current distribution patterns onto future climates. (2) Temporal variation in community structural variables can be correlated with variation in climatic variables and future changes deducted from them. (3) Experimental manipulations enable us to gain a mechanistic insight into plant community responses to climate change. These experiments can be done in the field where they manipulate larger

areas or focus on plant individuals or mesocosms in the lab. In the following, we will capitalize on field experiments but also briefly review our knowledge about spatial and temporal gradients in order to infer to which extent correlative approaches can substitute for experiments.

2.1 Space-for-Time Approaches

Space-by-time substitutions have been highlighted numerous times as convenient proxies to explore long-term ecological patterns like climate change (Cowles 1899; Whittaker 1975; Pickett 1989; Fukami and Wardle 2012). In these approaches, researchers collect information about the aspects of interest from various—typically distant—sites that are lined up along an ecological gradient. Inferences are then made to integrate the spatial variation in an ecological succession framework. For instance, wet, normal, and dry sites *become* wet, normal, and dry years, respectively (Pickett 1989). The rationale of this approach is also fundamental to the so-called bioclimatic envelope models (BEMs) which deduce climatic requirements for individual species from their current geographical range and project distributions under future climate scenarios, assuming that species will shift their ranges to track specific climatic conditions (Bakkenes et al. 2002; Peterson et al. 2002; Thomas et al. 2004; IPCC 2007a). Though such models have been criticized for not being mechanistic and for relying on a number of unrealistic assumptions (Pearson and Dawson 2003; Hampe 2004; Ibañez et al. 2006), they allow for the derivation of scenarios of change for many species and ecosystem properties based on very simple reasoning and at no experimental costs. Because evidence exists for range shifts already occurring (Walther et al. 2002; Parmesan 2006), a space-for-time approach may serve as a convenient first approximation for predicting ecosystem response to climate change.

For deserts, this approach has not been used explicitly to our knowledge. Instead, biogeographical principles have been used to provide a first insight into the potential trajectories of change. For example, at the global (Scheiner and Rey-Benayas 1994) and regional scales (Holzapfel et al. 2006), aboveground net primary productivity (ANPP), plant densities, and species richness decrease when moving from semideserts towards arid to hyperarid environments. Productivity patterns indicate that climate change expressed as increasing temperatures and decreasing precipitation will most likely result in lower biomass production, and fewer plant species or individuals. On the other hand, changes in plant community composition are more difficult to predict. Yet, the ubiquitous principle of ecology that "different species will respond differentially" will likely apply to climate change. Under the assumption that space can substitute for time, increasing aridity would favor species more adapted to arid conditions at the expense of "wet"-adapted ones.

Nonetheless, the reasoning behind current BEMs may be too simplistic. On the one hand, BEMs ignore the role of biotic interactions that may be disrupted under

climate change (Suttle et al. 2007; Tylianakis et al. 2008). In addition, they also overlook the fact that even species with a wide distribution range and an apparently large climatic niche may exhibit strong local adaptation and thus be prone to extinction in a changing world (Etterson and Shaw 2001). Vice versa, large plasticity in climate-related traits may render species less prone to extinction than predicted by simple correlative models.

The role of phenotypic plasticity and evolutionary processes is highlighted by many studies that have investigated trait variation of single species along aridity gradients. These include physiological and morphological traits in perennials such as modulations in leaf angles in *Encelia farinosa* along an aridity gradient in Chile (Squeo et al. 1994), compartmentalization of the mortality risk into independent physiological units in shrubs growing along gradients in North and South America (Schenk et al. 2008, R. Salguero-Gómez et al. unpublished data), or adaptive resource allocation in *Pinus ponderosa* along an abiotic gradient in Nevada (Maherali et al. 2002; Callaway et al. 2004). The extent to which this variation was due to plasticity or genetic differences has not been fully established. Ecotypic differentiation in climate-related traits has been found in numerous annual species inhabiting a steep aridity gradient in Israel, with clinal trends in seed dormancy and its heritability (Petrů and Tielbörger 2008; Lampei and Tielbörger 2010), flowering time (Liancourt and Tielbörger 2009; S. Hänel and K. Tielbörger unpublished data), survival rates and seed size (Metz et al. 2010), competitive ability (Liancourt and Tielbörger 2009), or reproductive allocation (Petrů et al. 2006).

Both plasticity as well as local adaptation will strongly affect the manner in which plants will respond to climate change. For example, if plasticity is large, climate change may have little effect on plant performance. If, on the other hand, the degree of local adaptation is high, climate change may lead to extinction of species even if they have a large distribution range, thus contradicting the predictions of BEM (Etterson and Shaw 2001). Based on the shortcomings of space-by-time substitutions carried out on single species, as reviewed above, we suggest that these approaches are not suited to generate predictions about temporal changes of desert vegetation under climate change (Dunne et al. 2004).

2.2 Time-for-Time Approaches

In addition to spatial gradients, researchers have used temporal gradients to infer relationships between ecosystem and climate variables. A significant body of paleoecological literature has studied the effects of past climate change over geological time and at large spatial scales (Cole 1990; Williams et al. 2000). However, these studies may not be too informative for our purposes because the rate of current climate change today is much greater than geological records show (IPCC 2007a), and because the resolution of paleoecological research with respect to plant community structure is rather limited.

Instead, observed temporal climatic variation in studies encompassing several years may be used as proxy for climate change effects. This approach appears particularly promising for arid environments, where temporal variation in precipitation and associated vegetation characteristics are typically large (Noy-Meir 1973). However, studies that have investigated climate–vegetation relationships over longer time periods for explicitly inferring potential climate change effects on desert plants are very rare. Vice versa, the body of literature about the effect of water availability on desert plants is extremely large and impossible to fully acknowledge here. Therefore, we summarize here only patterns of rainfall–vegetation relationships that can be generalized by pointing out representative studies that encompass longer time periods and that stem from different deserts of the world.

In general, the most consistent effect of changes in precipitation can be detected when examining plant productivity. Namely, biomass production in deserts is positively correlated with annual rainfall, exhibiting a linear relationship across a relatively large range of rainfall, but this relationship levels off at a certain point, likely due to nutrient limitation (Noy-Meir 1973; Lázaro et al. 2001; Ogle and Reynolds 2004). This pattern is also consistent with spatial gradients, albeit more consistent there (Sala et al. 2012), and therefore, it seems likely that changes in biomass will track precipitation amount and distribution in a future climate. In desert annuals, an increase in biomass is usually accompanied by higher fecundity (e.g., Kadmon 1994; Holzapfel et al. 2006), and several multiyear studies have shown that seed production is positively correlated with annual precipitation (Venable et al. 1993; Tielbörger and Kadmon 1997, 2000). The latter finding is interesting because it shows that annual plant densities, while being positively influenced by ambient rain through germination (Tielbörger and Kadmon 1997; Huxman et al. 2008), are also a function of previous year's rainfall amounts. In other words, wet years may not yield high plant densities if too few seeds were produced in the preceding years. Indeed, the relationship between ambient rainfall and annual plant abundance is relatively weak, and lag effects have been frequently observed (Venable et al. 1993; Huxman et al. 2008). Here, the relationship of abundance with rainfall in the preceding years appears to be the most common. Legacies of precipitation fluctuations are a frequent phenomenon in deserts (Sala et al. 2012). For example, perennial plant productivity responds to rainfall only with a certain lag (Ogle and Reynolds 2004), and response in abundance will be even further retarded. A general pattern seems that annuals respond more rapidly to rainfall variations than long-lived plants (Milton and Dean 2000).

Common to many desert ecological studies is not only a fast response, but also a high resilience of desert vegetation to drought (Bowers 2005). While drought years had strong immediate, negative effects on perennial plant survival and growth in a 7-year study in arid South Africa (Milton and Dean 2000), the recovery of the system was very fast. A 10-year study in the South African Karoo added another important aspect to the relatively low responsiveness of plant communities to rainfall variation in that grazing had an overwhelmingly large effect on vegetation development compared to a minimal effect of rainfall (Kraaij and Milton 2005).

Several long-term studies in the Sonoran Desert yielded an interesting and complex relationship between plant communities and rainfall. For example, in 76 years of observation by Bowers (2005), drought had a serious impact on mortality of several shrub species, but considerable mortality also occurred during extended wet periods, i.e., annual rainfall did not affect mortality in a consistent manner. This pattern was corroborated in a study using 60 years of photographic observations, where only a weak positive relationship between drought and mortality of woody plants and between wet periods and recruitment was detected (Turner 1990). A similar observation, albeit with the focus on recruitment, was made by Pierson and Turner (1998) in an 85-year demographic study of Saguaro cactus (*Carnegiea gigantea*) in the Sonoran Desert. Here, higher population regeneration of the cacti generally corresponded with relatively wet conditions, though extended periods of decline were also observed during relatively wet periods, indicating that other biotic or abiotic factors also determine recruitment success. Using 65 years of data from the Sonoran Desert to parameterize structural equation models, Butterfield et al. (2010) established the large importance of plant–plant interactions for desert plant demography. Namely, complex positive (facilitation) and negative (competition) interactions among adult shrubs and juvenile plants were even able to entirely decouple demographic processes and community abundance from precipitation patterns.

The role of soil depth for determining shrub responses to rainfall was highlighted by Browning et al. (2012) in a 71-year study in the Chihuahuan Desert by using historical imagery. In that study, shrub growth was positively correlated with above-average rainfall years in regions characterized by deep soils, but shrub recruitment was larger in drought years on shallow soils. Such unexpected patterns can actually be frequently found in arid environments, and two more examples should be mentioned here. Using 16 years of rainfall records and normalized difference vegetation index (NDVI) data for two sites in semiarid Chile, de la Maza et al. (2009) predicted that El Niño years with very large rainfall amounts will result in phenological changes of the vegetation, but that decreasing precipitation will have little effect on primary production. A particularly puzzling result was obtained by Kimball et al. (2010) for Sonoran Desert winter annuals. The authors showed that the observed warming and drying in the past 25 years led to an increase in cold-adapted plant species. This pattern could be explained by the fact that not only the amount of rainfall decreased and temperature increased, but especially rainfall was shifted to a later point in time in the growing season. Due to that temporal shift, the main germination period fell into a colder season than 25 years ago, thus favoring cold-adapted species over warm-adapted ones.

We argue that the relationship between vegetation characteristics and temporal variation in precipitation is rather complex, and often associated with a lag phase. Therefore, patterns observed from temporal variation need to be supported by experimental evidence.

2.3 *Evidence from Field Experiments*

Experimental information (Fig. 2) about effects of changes in precipitation and temperature on arid plant populations and communities is extremely scarce. For example, Miranda et al. (2011) published a review of field experiments manipulating precipitation in dry ecosystems, and found 22 studies from only seven experimental systems altogether. Out of these, only two systems (six studies) were conducted in subtropical deserts or semideserts, and in only one of them plant community structural components (diversity, biomass production) were used as response variables (Miranda et al. 2009a, b, 2011). The lack of experiments is also highlighted in a more recent review on precipitation experiments worldwide (Beier et al. 2012). A main conclusion of that study is that deserts are particularly understudied with respect to experimental rainfall manipulation in the field.

With deserts being at the forefront of climate change (IPPC 2007a), the fact that so little is known about them is rather surprising. The reasons for the scarcity of studies can be manifold and most likely include logistic challenges due to remoteness of desert environments from research institutions (Martin et al. 2012), or limited funds to support extensive infrastructure in many arid to semiarid countries. Furthermore, climate change projections for arid environments that stem from Global Circulation Models (GCM) may show considerable disagreement and range from rather limited impact to increased or decreased precipitation (Lioubimtseva and Adams 2004). This hampers our ability to design studies that manipulate climatic variables within "realistic" limits. Consequently, experiments that were inspired by robust GCMs or downscaled regional models are rather rare (but see Miranda et al. 2011; Salguero-Gómez et al. 2012; Sternberg et al. 2011; R. Salguero-Gómez et al. unpublished data).

Due to the scarcity of experiments, we have subjectively relaxed our search criteria, to obtain at least a basic insight into the possible response of desert vegetation to projected climatic shifts. In particular, the present review includes studies with the following characteristics:

- Studies that were conducted in semiarid areas (sensu Meigs 1953, Fig. 1)
- Studies testing for the effects of elevated CO_2 concentrations and/or precipitation manipulation
- Greenhouse experiments which manipulated entire communities or dominant plant species and thus may have larger implications for overall vegetation response to change
- Previously unpublished data from an own ongoing experiment along an aridity gradient in Israel (Sternberg et al. 2011)

2.3.1 Impacts of Elevated CO_2

While the potential impact of climate change may theoretically be inferred from natural climatic gradients or climatic variations in time, the effects of elevated CO_2 can only be assessed experimentally. It has been suggested that arid environments

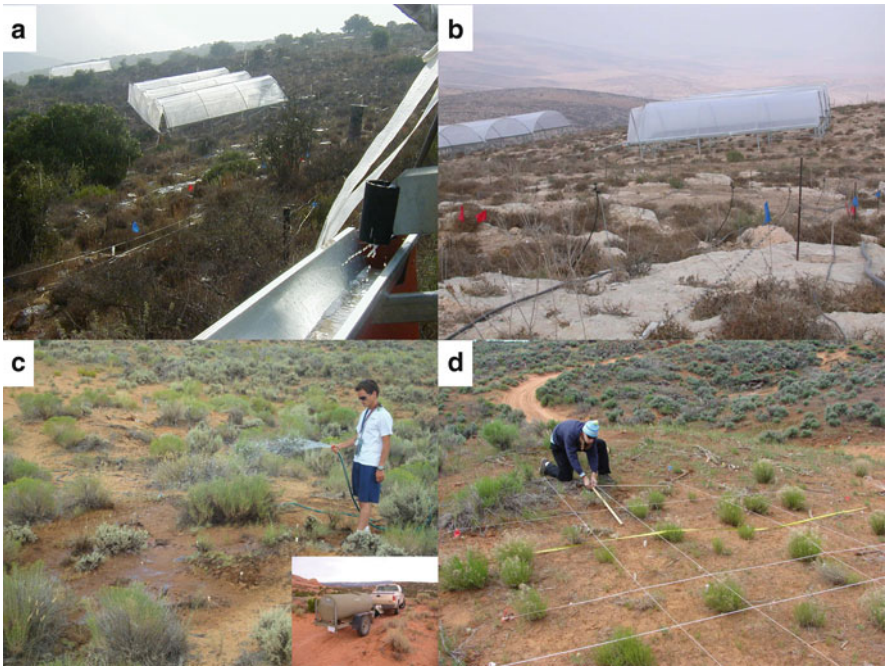

Fig. 2 In situ experimental approaches provide us with the most reliable information regarding how desert ecosystems will respond to projected shifts in precipitation and increases in temperature. *Panel* (**a**) and (**b**) depict rainout shelters used to mimic projected droughts in Mediterranean (**a**) and semiarid (**b**) environments in Israel. In some other arid regions, increases in precipitation are projected, like in the Great Basin Desert [*panel* (**c**) and (**d**)]. In those cases, pulses of precipitation are simulated by transporting significant amounts of water to the field [*insert* in *panel* (**c**)]. The subsequent long-term censuses [*panel* (**d**) provide researchers with firsthand evidence of the effects of climate change on the desert vegetation (Photo credits: (**a**) & (**b**) by K. Tielbörger; (**c**) & (**d**) by R. Salguero-Gómez)

may be particularly responsive to increased concentrations of CO_2 because of their inherent water limitation and the potential for CO_2 to increase water use efficiency (Bazzaz 1990; Melillo et al. 1993). Therefore, a number of greenhouse studies and a few field studies have investigated CO_2 effects on arid land plants, albeit mostly in isolation and with little respect to changes in temperatures and precipitation (Lioubimtseva and Adams 2004). Furthermore, caution is needed in interpreting all available findings, as none of the field experiments has been running sufficiently long to exclude the possibility that short-term CO_2 effects may decline or disappear, as shown in non-arid biomes (Lioubimtseva and Adams 2004).

A particularly relevant set of studies were conducted by Grünzweig and Körner, who exposed mesocosms of soil and annual plant seeds from a species-rich semi-arid environment in the Negev to two scenarios of enhanced CO_2 (2000a, b, 2001). Their main result was that the overall response of the plant communities was much smaller than expected for arid systems, as also highlighted previously (Bazzaz

1990; Melillo et al. 1993). Namely, the recorded responses by Grünzweig and Körner were mostly due to only one or very few species (1–5 out of 32). Legumes were most prone to respond positively to elevated CO_2 in terms of biomass production and fecundity, while forbs and grasses exhibited no detectable response.

Along similar lines, a controlled glasshouse experiment with three Mojave desert grasses indicated that C4 grasses and an annual C3 grass showed rather limited response to elevated CO_2 in above- and belowground growth, while some increase in biomass could be detected in a perennial C3 grass (Yoder et al. 2000). The extent to which such controlled experiments with few species can be extrapolated to entire plant communities remains to be explored. Furthermore, the aforementioned study was conducted over a single year, and a longer period of manipulation may yield different results. For example, after exposing a semiarid grassland community in the North American Great plains to five years of elevated CO_2, Morgan and colleagues (2007) observed a positive effect on biomass of C3 grasses. Though overall diversity remained unaffected, species composition changed over time with C3 plants (grasses and shrubs) profiting from CO_2 enrichment at the expense of C4 grasses. Based on these patterns, Morgan et al. (2007) concluded that shrub encroachment, a major threat to the provisioning of ecosystem services in drylands, may be promoted by elevated CO_2.

The species specificity of CO_2 effects was corroborated by a study in which Mojave Desert plant communities were exposed to increased levels of CO_2 during one wet and one dry year (Smith et al. 2000). Here, elevated CO_2 resulted in larger productivity of shrubs and annuals and in higher annual plant density only in the wet year and more so under shrub canopies, i.e., in patches with possibly higher soil nutrient content. This indicates that CO_2 may have positive effects only when other resources such as water and nutrients are less limiting. While this relationship has been suggested previously for nitrogen, the combined effect of CO_2 and precipitation was surprising because CO_2 may offset the negative effects of drought, thus suggesting that elevated CO_2 should be more, rather than less effective when water is scarce. Another conclusion drawn by Smith et al. (2000) was that invasive annuals may profit from climate change at the expense of native plants, because the former were more responsive to elevated CO_2 in terms of abundance, productivity, and fecundity.

In summary, CO_2 effects on desert vegetation are equivocal, and either positive or neutral effects of elevated CO_2 have been reported. CO_2 effects are highly species specific or depend on interactions with other resources making generalizations very difficult. However, we may conclude from the existing experimental evidence that the impact of elevated CO_2 on plant community structure, productivity, and physiological parameters is smaller than expected for arid ecosystems.

2.3.2 Manipulation of Amount and Distribution of Precipitation

Water is the main limiting factor for plant growth in deserts (Noy-Meir 1973), highlighting the extraordinary importance of precipitation changes for structure and function of arid plant communities. Consequently, studies manipulating rainfall are

more common in arid lands than in other systems, e.g., tundra and alpine systems, where temperature manipulations are most common (Walker et al. 2006).

The study with the longest period of rainfall manipulation published to date was conducted in a semiarid steppe in Colorado (Evans et al. 2011). In this experiment, extreme drought scenarios of minus 50 % and 75 % ambient rainfall, respectively, were implemented during an 11-year study period. Despite the large duration and the extreme treatments, the systems responded very slowly. For instance, the first marginal response of the plant community was detected after 4 years of experimental drought, and clear effects such as a decrease in total cover and in cover of perennial grasses for the benefit of ruderal species emerged only after 7 drought years. While species composition changed during the course of the study, species richness was, on average, unaffected by drought even if in some years drought plots differed from controls. Therefore, the lesson from this study is twofold: the semiarid plant communities resisted rather long periods of drought (4–7 years), but shifts in community structure and productivity emerged after a lag phase which would have gone unnoticed in short-term studies. Interestingly, the two drought treatments did not differ consistently in their effect, indicating that the duration of the drought was more important than its intensity.

A lag phase in response to rainfall manipulation was also observed in two other long-term studies, one conducted for 7 years in a cold desert steppe (Great Basin, US; Bates et al. 2006), and the other one in a hot semidesert in Spain (5 years, Miranda et al. 2011). In the Great Basin, the temporal distribution of rainfall was manipulated such that either spring or winter rains were decreased compared to controls. The vegetation responded to these treatments mostly after about 4 years, with herbaceous plants exhibiting a complex response to shifting rainfall patterns, but the dominant plant species (*Artemisia tridentata*) not responding in terms of abundance (Bates et al. 2006). In the field experiment by Miranda et al. (2011), a 5-year manipulation of rainfall amount (−30 % from the control conditions) and frequency was imposed on a semiarid plant community in southern Spain. This experiment mimicked climate scenarios for the Mediterranean region, which predict a decrease in average rainfall and an increase in the frequency of extreme events (IPCC 2007a). The main finding of this study was that the plant communities were highly resistant to change, especially in the first years of the manipulation. However, after a lag phase, some isolated effects of experimental drought were detected. Namely, biomass and CO_2 flux of one annual grass species were reduced after 2 years in droughted plots, and ANPP decreased after 4 years of manipulation. Some increase in biomass of an annual legume was detected after 4 years of altered seasonal rainfall distribution. However, the overall response in plant physiological and performance parameters was rather small, suggesting again a relatively high resistance to climate change.

The same research team also performed shorter experiments in semiarid Spain, where frequency and timing of rainfall were manipulated simultaneously (Miranda et al. 2009b), and then the plant communities emerging from soil cores were studied. These experiments yielded a highly complex response of annual plants to different levels and seasonality of rainfall. In general, any deviation from "average"

conditions, namely, decrease in amount and number of rainfall events and increase in within-season variability of rain, reduced the density of plant individuals and their biomass. This result points to a certain sensitivity of semiarid communities to climate change. These findings were partly corroborated by field experiments nearby (Miranda et al. 2009a), where rainfall amounts were reduced by 50 %. However, in that case the reduction combined with a change in frequency of rain did not have any detectable effects on biomass and abundance. Therefore, the overall findings by Miranda et al. (2009a, b, 2011) suggest a certain degree of resistance of desert plant communities to increasing aridity.

A similar conclusion has been recently drawn by Suazo et al. (2012), who performed an irrigation experiment in the Mojave Desert and examined the response of native and nonnative winter annual species to full-factorial combinations of increased rainfall and disturbance. Though no drought treatment was applied, the range of sites and precipitation scenarios allowed some inference about the plant community response to changing rainfall. Two invasive annuals responded positively to irrigation in reproductive allocation (*Brassica tournefortii*) or density (*Schismus arabicus*), when irrigation was combined with disturbance. A native *Eriophyllum* species responded positively to water supplementation, too. From an overview of native species densities (no community-level analysis), the authors conclude that native species composition did not change with the rainfall manipulations. Another irrigation experiment in the USA was performed in a semiarid Californian grassland which is dominated by exotic annuals (Harpole and Potts 2007). In that region, climate scenarios project an increase in rainfall, and so this experiment combined irrigation with nitrogen addition treatments. Interestingly, the effect of water supplementation on the plant communities (diversity and ANPP) was negligible with a single effect of irrigation on biomass when nitrogen was supplemented. This example indicates that when more than one resource is limiting, as is characteristic for deserts (Noy-Meir 1973), an increase in one crucial resource may not be sufficient to elicit a community response.

A two-species study by Reynolds and colleagues (1999) may provide a first explanation for the apparently limited response of desert plants to increased aridity. In this study, rainfall was reduced in semiarid regions in Southwestern USA and the physiological responses of two dominant shrub species to summer or winter drought were observed. Though some reduction in photosynthetic activity and growth in response to drought occurred, one species (*Larrea tridentata*) shifted its activity to periods of higher water availability, and both species compensated for some negative impacts of drought. These findings suggest an adaptive capacity in dryland plants, in this case through a plastic response to varying rainfall conditions.

The most compelling evidence to date for the apparent resistance of dryland communities to increasing aridity stems from our own work that was conducted along a steep aridity gradient in Israel (Sternberg et al. 2011). Though this study encompassed arid (90 mm average annual precipitation) to mesic Mediterranean (780 mm) conditions, the rainfall manipulations inspired by regional climate change scenarios (Smiatek et al. 2011) were performed only in a semiarid (280 mm average rainfall) and a Mediterranean site (550 mm). Figure 3 shows a

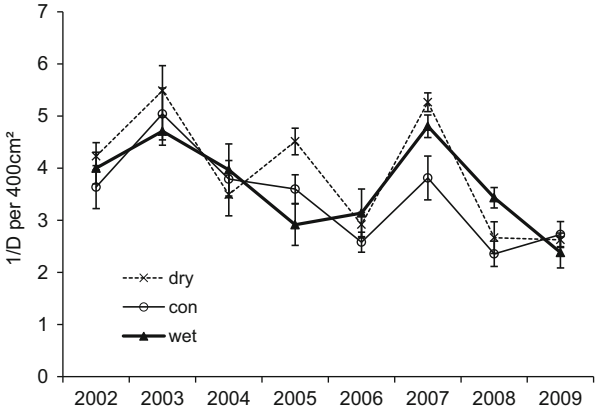

Fig. 3 Mean (±SE) plant diversity (Simpson's index 1/*D*) in permanent 400 cm^2 quadrats monitored in semiarid plant communities in Israel. The natural communities were subjected to 7 years of drought (dry—minus 30 % precipitation), irrigation (wet—plus 30 % precipitation), and natural (control) rainfall conditions in a semiarid site in Israel. The first year represents the baseline without manipulation for comparative purposes. There were no significant effects of rainfall treatments on plant diversity, albeit in some years, diversity differed among quadrats (year × treatment interaction: $P = 0.014$). The drought treatment mimicked regional climate change scenarios (Smiatek et al. 2011)

representative finding of that long-term study (K. Tielbörger et al. unpublished data): annual species diversity did not change after 7 years of climate manipulation. This was also true for biomass, densities, species richness, and multivariate community structure (K. Tielbörger et al. unpublished data), and the same findings still held after 10 years of precipitation manipulation (M. Bilton, unpublished data). Interestingly, temporal variations in all these parameters were very large (Fig. 3), as were also the differences in biomass and species diversity from adjacent sites (Holzapfel et al. 2006; Sternberg et al. 2011), indicating that neither space nor time could substitute for the experimental evidence.

2.3.3 Impacts of Increased Temperatures

Unlike in alpine regions or tundra ecosystems (Walker et al. 2006), warming experiments in deserts are mostly entirely lacking (but see Collins et al. 2010), probably due to the apparently overwhelming importance of water in plant performance relative to temperatures. However, warming has been shown to be beneficial in cold deserts that are seasonally limited by low temperatures (Lucas et al. 2008). Also, in regions where climate scenarios predict an increase, rather than a decrease in precipitation (e.g., in California, Harpole and Potts 2007), increased temperatures may buffer the effects imposed by rainfall changes. In the opposite cases, i.e., (hot) deserts with decreasing rain, increased temperatures would aggravate the lack of water under climate change by increasing evapotranspiration.

Therefore, experimental manipulations of both precipitation and temperature are of large ecological importance. The results of Collins et al. (2010) may be surprising at a first glance because nighttime warming in the Chihuahuan Desert increased productivity of dominant plant species. However, this study was conducted in a single year, which was extraordinarily wet, and thus increased evapotranspiration was offset by higher precipitation. This finding, though, is unlikely to be representative for the response of arid systems to warming.

3 Discussion

Overall, the available evidence points towards a great deal of resilience in dryland plant species to climate change. This may appear counterintuitive: one may expect deserts to be particularly vulnerable to scenarios of warming and decreasing precipitation since its biota appears to already live at their biological limit. Moreover, such a feeling is in agreement with vulnerability assessments made by the intergovernmental panel for climate change (IPCC 2007a). However, these assessments are based on the logic of bioclimatic models, which map future species distributions and ecosystem properties unto future climatic maps based on the current co-occurrence of certain systems with climate (e.g., Bakkenes et al. 2002; Thomas et al. 2004; IPCC 2007a). In that respect, BEMs are based on the same logic as the space-for-time substitution approach that uses spatial correlations between ecosystem properties and climatic variables as a proxy for prospective changes in time. BEMs have been widely criticized because of a number of unrealistic assumptions about species distributions (see also Sect. 2.1). For example, these apply commonly simplistic "full dispersal" or "no dispersal" scenarios for species migration abilities (Guisan and Thuiller 2005), they ignore other abiotic factors apart from climate and the crucial role of biotic interactions (Suttle et al. 2007; Tylianakis et al. 2008), and thus they assume a perfect match between fundamental (climatic) niche and realized niche. Finally, these models ignore microevolutionary processes such as the potential to adapt to change (Jump and Peñuelas 2005), and they treat species as undifferentiated entities comprised of individuals with broad tolerance levels, instead of locally adapted ecotypes with much narrower tolerances (Etterson and Shaw 2001).

We have argued that the space-for-time approach is not the strongest approach in climate impact research, as differences among species or communities along climate gradients are the result of long-term evolutionary processes that are unlikely to match the current speed of climate change. Therefore, an important result of our review is the great disparity between the conclusions drawn from space-for-time approaches and the findings of experiments. Namely, the spatial gradients suggest that in a scenario of increasing aridity, plant community parameters should follow a trajectory towards conditions prevailing under currently more arid conditions. For example, ANPP, density, and diversity should decrease with increasing aridity, and species composition should change because species

adapted to wetter conditions would go extinct or migrate to a wetter climate. Unfortunately, such a rationale seems intuitive and has therefore been the basis of BEMs, which in turn influenced assessments of vulnerability of ecosystems to climate change (IPCC 2007a). As we have shown here, these assessments, which draw a bleak future for arid ecosystems, may be more pessimistic than suggested by experimental evidence.

We propose that a main reason for the apparent resilience of desert vegetation to climate change resides in the very evolutionary history of desert plants. For one thing, plants are stuck in their location and thus their stress avoidance mechanisms (e.g., seedbank (Cohen 1966), dieback (Kozlowski 1973); see below) are likely more important to their fitness than those of mobile organisms. Plants that are native to deserts must also have adapted to the high variability in rainfall conditions that characterizes arid ecosystems (D'Odorico and Bhattachan 2012). Projected and experimentally simulated scenarios of change mostly lie within the boundaries of rainfall ranges that these plant communities frequently experience even under past/current conditions. As a consequence, a drastic response appears unlikely for desert plants.

Interestingly, even very drastic manipulations such as the −75 % ambient rainfall scenario imposed by Evans et al. (2011) did not cause any detectable response of the dominant plant species for up to 7 years. Despite the high natural variability in rainfall, this may still appear surprising, because such an extreme and extensive drought in severely water-limited areas should impose a much larger stress on the plants than in humid regions. However, this hand-waving evaluation ignores the fact that stress or limitation should be defined as the deviation from an optimum or average state (Körner 2003), and this average is clearly different for desert plants than for plants inhabiting humid areas. In particular, desert plants exhibit a large range of adaptations that enable them to cope with the stress conditions typical for deserts such as high temperatures and radiation, low (average) water and nutrient availability, and high variability in resource availability. Adaptations to lack of water and high temperatures are commonly found in desert plants and include succulence (van Willert et al. 1992), water-saving photosynthetic pathways (Cushman 2001), reduction of leaves and stems (Gibson 1998; Ewers et al. 1992), or avoidance strategies such as ephemeral occurrence of aboveground plant parts (Ewers et al. 1992; Bowers and Turner 2001; Salguero-Gómez and Casper 2011a) and fine root production (Salguero-Gómez and Casper 2011b). Annual plants are particularly well adapted to arid conditions as they exhibit life history strategies that enable them to avoid the drought periods and exploit, on average, more favorable conditions. For example, seed dormancy helps avoiding extinction in drought years by spreading the risk over many consecutive years (Cohen 1966). This behavior and other "bet-hedging" strategies such as large seeds (Rees 1996), seed dimorphisms (Venable 1985), or dispersal in space (Venable et al. 2008) may also contribute to buffering the immediate response of annuals to climate variation. A modeling study that was based on long-term experimental and demographic data from two deserts supported this view (Salguero-Gómez et al. 2012). Extinction risks for two desert plant species, an annual from a hot desert and

a perennial from a cold desert, were found to be very small, and this resistance was attributed to risk reducing mechanisms. In summary, adaptations to stressful and highly variable climatic conditions may explain why desert plants appear to be particularly resistant to climate change.

Nevertheless, even if the general pattern suggests little response of desert plants to climate change, there are conditions under which a response is more likely to be noticed. Most importantly, long-term experiments were much more likely to detect a response to precipitation or CO_2 manipulation (Bates et al. 2006; Evans et al. 2011; Miranda et al. 2011, but see Fig. 3). Secondly, irrigation generally appears to have larger effects on plant communities than drought across all experiments. Also, data from our own study suggest that irrigation had some positive effect on annual biomass production (K. Tielbörger et al., unpublished data), while experimental drought caused no response in any of the measured variables (Salguero- Gómez et al. in preparation vs. Lucas et al. 2008, respectively, for experimental examples on the very same species with diametrically opposed treatment effects). In addition, there seems to be a difference among response variables with biomass being more likely to respond to rainfall manipulation than plant density or species diversity. Also, species composition did change in some cases in response to CO_2 or precipitation manipulation due to species-specific responses, but species diversity was generally unaffected. Moreover, there seemed to be a trend to larger responsiveness of short-lived plants (coined mostly "weeds" or "exotic annuals") compared to (native) perennials, especially in studies from the USA (e.g., Smith et al. 2000; Suazo et al. 2012). However, our own findings from native annual communities which exhibited a high resistance to precipitation change (K. Tielbörger et al., unpublished data) indicate that these patterns cannot be generalized. Finally, precipitation appears to be more important than temperatures and CO_2 concentration in determining desert plant responses to change. However, this conclusion needs to be taken with caution due to the low number of studies manipulating CO_2 (four studies) and temperatures (one study).

A main lesson from our review is that long-term experiments (>10 years) are particularly important in highly variable desert ecosystems, as responses are unlikely to be detectable within few years. Unfortunately, such experiments translate into long-term commitments that go against economic and logistical limitations. A useful substitute for in situ manipulation of climatic conditions could be experiments such as the one performed by Link et al. (2003) in a semiarid environment. In it the authors manipulated climate indirectly by reciprocal transplants of soil cores and a dominant grass along a climatic gradient. Observations of plant physiological parameters indicated no effect of indirect warming and drying on performance of the focal grass (*Poa secunda*). Approaches such as this one may be an elegant, feasible, and less costly alternative to in situ climatic manipulations. If reciprocal transplants are combined with soil transplants, they may also avoid confounding sources that may co-occur if only seeds or plants were relocated (Petrů and Tielbörger 2008). However, such experiments are ultimately limited to one or few growing seasons, which may again limit the conclusions that can be drawn from them.

4 Conclusion

There is a striking lack of desert field experiments manipulating climatic variables in a realistic manner and, more importantly, over a meaningful time period. Furthermore, experiments in strictly speaking *arid* environments (sensu Meigs 1953) are virtually absent on our map of study sites (Fig. 1). Finally, rainfall manipulations dominate the literature, where simultaneous manipulation of rainfall (amount and frequency), temperatures, and CO_2 concentrations would be highly desirable.

Calls for long-term studies have been made numerous times in ecological settings (Knapp et al. 1998; Lindenmayer et al. 2012). Despite this apparently predictable and possibly dull conclusion, we would like to stress that the need for long-term scientific commitments—on the side of the funding agencies as well as on the research teams—is particularly pressing for arid environments for the following reasons. First, dryland environments have been classified as particularly vulnerable to global change because they are expected to face a combination of large climatic changes and increasing human pressure (Sala et al. 2000; Thornton et al. 2008). However, such classifications are not yet based on sound experimental evidence, which seriously hampers our ability to develop adaptive management. Secondly, a main feature of arid ecosystems is not only the lack of water, but the high variability and unpredictability of within- and between-year climatic conditions (D'Odorico and Bhattachan 2012). Therefore, studies spanning over too few years will not be able to distinguish the signal of climate change from the noise of natural climatic variation. Moreover, specific adaptations of desert plants to variable and sometimes stressful conditions may render desert plant communities quite resilient to short-term changes. These characteristic features may be taken as a carefully optimistic note, though the lack of long-term data should rank as a top priority of the research agenda of dryland scientists.

Despite this cautiously positive scenario for desert plant communities under climate change, global and regional classifications have placed drylands among the most vulnerable (Sala et al. 2000; Thuiller 2007; Thornton et al. 2008). These classifications are based on a combination of assumptions including (1) climate scenarios which predict, for many desert regions, a decrease in precipitation, an increase in temperatures, and more frequent extreme events; (2) increasing human pressure due to population growth; and (3) a low adaptive capacity of many societies living in arid to semiarid regions.

The evidence gathered here indicates that the impact of climate change on deserts may be overestimated and ignores the adaptive capacity of natural systems to a large range of climatic conditions. However, the more tangible threats to the functioning of these unique ecosystems, such as changes in land use and overexploitation of resources, will remain to be very important. For example, humans have also developed strategies to cope with the large fluctuation in conditions in deserts. Some of these strategies include pastoralism (Meze-Hausken 2000) and the plastic usage of temporarily emerging biotic resources (Morton et al. 2011). However,

these strategies are no longer sustainable as the population in some arid lands may double within a few decades (IPCC 2007a). This has put drylands under an unprecedented level of human pressure. Some examples of the increasing exploitation of drylands' natural resources include overgrazing by livestock (e.g., International Livestock Research Institute 2006; Thornton et al. 2008) and exploitation of minerals and oil deposits (White and Nackoney 2003; OPEC 2010), both of which have already led to significant erosion and man-induced degradation of drylands (Millennium Ecosystem Assessment 2005; Romm 2011). Therefore, while climate change alone may pose a smaller threat to arid ecosystems than previously assumed, other drivers such as land use change will need to be addressed for sustaining these unique ecosystems in an era of global change.

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