Editorial

The *New Phytologist* Tansley Medal

New Phytologist was founded by Sir Arthur Tansley and was first published in 1902. The long and independent history of the journal has allowed a positive inflow of revenue that is managed by the New Phytologist Trust and which is ploughed back into the scientific community to foster plant science (for a brief history of *New Phytologist* see Lewis & Ingram, 2002). The Trust currently finances two scientific meetings a year (e.g. Bruns & Kennedy, 2009; Harvey & Strauss, 2009) in addition to providing support for other meetings and activities (see http://www.newphytologist.org). A new innovation from the Trust is an annual competition for the *New Phytologist* Tansley Medal, specifically aimed at plant scientists in the early stages of their career. The plant scientists are required to submit their best research, either as a research article or as a small review, in a global competition for the award of a £2000 prize, in addition to publication of the work and an editorial comment in *New Phytologist*. Details of the practicalities can be found on the New Phytologist Trust website at http://www.newphytologist.org/tansleymedal.htm.

The *New Phytologist* Tansley Medal competition is a two-round process. In the first round, candidates submit an extended abstract of their work, together with their *curriculum vitae* and a reference from a scientist who has agreed to support the application. The number of applications at this stage is large and they are sifted by two Editors to produce a short list of candidates (seven candidates for the 2009 medal) who progress to the second round. Those who make it to this stage are requested to submit a complete manuscript, which is then peer-reviewed in the usual way. In addition, the Referees and Editors are asked for their assessments of the suitability of the manuscript for the Tansley Medal. Two Editors then make the final choice of the medal award.

This year we are delighted to award the first Tansley Medal to Steven Spoel from the Institute of Molecular Plant Sciences at the University of Edinburgh, UK, for his paper on *Post-translational protein modification as a tool for transcription reprogramming* (this issue, pp. 333–339). As the title suggests, the manuscript deals with the control of transcriptional reprogramming. This process contributes to, for example, the co-ordinated changes in gene expression that occur during development or during adaptation to environmental stress. Spoel and his co-authors focus on the contribution that protein modification makes to the control of transcriptional reprogramming. In a lucid and highly accessible account they discuss how the activation and repression of genes can be achieved by phosphorylation, ubiquitinylation, *S*-nitrosylation and disulphide-bonding of transcriptional activators, co-activators and repressor proteins. The Minireview provides examples of how these processes operate and their significance in relatively well-understood systems such as yeast. However, the main focus is on the plant immune response and especially on how modification of the immune co-activator protein NPR1 can control its localization and abundance in plant cells. The result is a highly authoritative review of an emerging topic of fundamental importance to our understanding of plant cell biology.

New Phytologist also publishes papers of candidates in the final short list, following successful review. Congratulations are due to these candidates who have provided manuscripts on a wide range of topics in plant sciences and which can be read in this issue.

- Ward Capoen, Sesbania rostrata: a *case study of natural variation in legume nodulation* (pp. 340–345)
- Colleen Iversen, *Digging deeper: fine-root responses to rising atmospheric CO_2 concentration in forested ecosystems* (pp. 346–357)
- Katherine McCulloh, *Moving water well: comparing hydraulic efficiency in twigs and trunks of coniferous, ring-porous, and diffuse-porous saplings from temperate and tropical forests* (pp. 439–450)
- Rebecca Mosher, *Maternal control of Pol IV-dependent siRNAs in* Arabidopsis *endosperm* (pp. 358–364)
- Nicholas Rouhier, *Plant glutaredoxins: pivotal players in redox biology and iron–sulfur center assembly* (pp. 365–372)

Thank you to the many applicants who submitted extended abstracts and also to those Editors and Referees who contributed in full to this first enterprise for the *New Phytologist* Tansley Medal.

F. Ian Woodward
Editor-in-Chief, *New Phytologist*
(email f.i.woodward@sheffield.ac.uk)

Alistair M. Hetherington
Tansley review Editor, *New Phytologist*
(email alistair.hetherington@bristol.ac.uk)

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McCulloh K, Sperry JS, Lachenbruch B, Meinzer FC, Reich PB, Voelker S. 2010. Moving water well: comparing hydraulic efficiency in twigs and trunks of coniferous, ring-porous, and diffuse-porous saplings from temperate and tropical forests. *New Phytologist* **186**: 439–450.

Mosher RA. 2010. Maternal control of Pol IV-dependent siRNAs in *Arabidopsis* endosperm. *New Phytologist* **186**: 358–364.

Rouhier N. 2010. Plant glutaredoxins: pivotal players in redox biology and iron–sulphur centre assembly. *New Phytologist* **186**: 365–372.

Spoel SH, Tada Y, Loake GJ. 2010. Post-translational protein modification as a tool for transcription reprogramming. *New Phytologist* **186**: 333–339.

Key words: Arthur Tansley, fine-root responses, glutaredoxins, legume nodulation, *New Phytologist* Tansley medal, plant hydraulic efficiency, small interfering RNAs (siRNAs), transcription reprogramming.

Commentary

The mechanisms of carbon starvation: how, when, or does it even occur at all?

Recent observations of increasing vegetation mortality events appear to be a result of changing climate, in particular, an increase in the frequency, length and intensity of droughts (e.g. Allen *et al.*, 2010). The threat of widespread increases in future mortality has rekindled interest in the mechanisms of plant mortality and survival because we do not yet understand them well enough to confidently model future vegetation dynamics (Sitch *et al.*, 2008). In this issue of *New Phytologist*, Sala *et al.* (pp. 274–281) provide a viewpoint on the 'carbon (C) starvation hypothesis' (McDowell *et al.*, 2008). Their viewpoint is invaluable for stimulating our field to explicitly refine our definitions and identify the key experiments needed to understand mechanisms of vegetation survival and mortality. Two important conclusions of their paper were that mortality can occur at nonzero carbohydrate levels and that careful experiments focused on the explicit mechanisms of C starvation, as well as on partitioning the roles of hydraulic failure and C starvation, are needed to understand the physiological underpinnings of how plants die. We applaud these conclusions, and agree that hasty acceptance of any hypothesis before adequate testing is foolish. In this commentary, we highlight some of the valuable ideas from Sala *et al.* and provide additional comments that we hope will prompt careful future tests on the mechanisms of plant mortality.

'The paucity of studies that quantified mortality forces scientists to use data from nonmortality studies to develop hypotheses … we do this at the risk of confusing stress responses with mortality mechanisms.'

When the C-starvation hypothesis was proposed (McDowell *et al.*, 2008), it represented an attempt to summarize and interpret the existing literature on vegetation mortality, of which there was a wealth of indirect studies, but a paucity of true, mechanistic tests. The original formulation of the hypothesis suggested that stomatal closure minimizes hydraulic failure during drought, causing photosynthetic C uptake to decline to low levels, thereby promoting carbon starvation as carbohydrate demand continues for maintenance of metabolism and defense. The plant either starves outright, or succumbs to attack by insects or pathogens, whichever occurs first. By contrast, failure to maintain xylem water tension lower than its cavitation threshold results in embolisms, which, if unrepaired, can eventually lead to widespread hydraulic failure, desiccation and mortality. We hoped that the C-starvation and hydraulic failure hypotheses would generate discussion and new ideas; and

indeed, as summarized by Sala *et al.*, active discussion is taking place. A primary conclusion from the discussion is that we need clarification of the various mechanisms by which C starvation can occur, if it occurs at all.

Plants maintain metabolism through respiratory processes that consume carbohydrates, and in doing so their C budgets must obey the law of conservation of energy, that is, respiration (mols per plant) = photosynthesis + carbohydrate storage − growth. Therefore, if not all carbohydrates are available for metabolism during drought, this will accelerate C starvation by reducing the storage pool available for respiratory metabolism. The current evidence is mixed regarding metabolic limitations to utilize carbohydrates. We reviewed the four carbohydrate studies cited by Sala *et al.* that included plant mortality, as opposed to publications in which seasonal carbohydrate analyses were performed on plants that did not die (representing 4 out of 16 (or 25%) of the studies on carbohydrate cited by Sala *et al.*), and agree that no clear pattern of carbohydrate content and mortality emerges. Mortality at nonzero carbohydrates could simply be a result of mortality via other mechanisms, such as hydraulic failure. However, mortality at nonzero carbohydrate contents could also be a result of C starvation because of the increased use of sugars for osmotic balance during drought. These sugars may be unavailable for other metabolic maintenance processes (Chaves *et al.*, 2003; Bartels & Sunkar, 2005; N. G. McDowell & J. Amthor, unpublished; Sala *et al.*). Consistent with this, a classic paper by Marshall & Waring (1985) demonstrated that shaded trees consumed all of their starch pools and subsequently died (J. D. Marshall, pers. comm.), but their sugar pools remained well above zero. Elevated temperatures accelerated depletion of the starch pools and subsequent mortality, consistent with the recent results from Adams *et al.* (2009). Given the current available evidence, it is quite possible that not all carbohydrates can be utilized, particularly during drought.

The paucity of studies that have quantified mortality forces scientists to use data from nonmortality studies to develop hypotheses (i.e. McDowell *et al.*, 2008; Sala *et al.*); however, we do this at the risk of confusing stress responses with mortality mechanisms. Particular to C starvation, the literature on the carbohydrate patterns of plants that did not die may not be evidence against C starvation. In fact, these data, along with the widespread evidence that plants minimize C loss and maximize C gain during drought (reviewed by McDowell *et al.*, 2008; Sala *et al.*; and many others), support the critical role of carbohydrate balance in avoiding mortality. During drought, carbohydrates accumulate because growth declines faster than photosynthesis (reviewed by N. G. McDowell & J. Amthor, unpublished). This is driven not only by mass balance, but also through feedforward signaling in response to carbohydrate availability that down-regulates growth and respiration and up-regulates storage in direct response to depletion of photosynthate and starch (Smith & Stitt, 2007; Gibon *et al.*, 2009). The carbohydrate concentration of tissues should only decline when the availability of C from photosynthesis plus storage does not equal C consumption to maintain metabolism (Marshall & Waring, 1985; N. G. McDowell & J. Amthor, unpublished). Therefore, the primary driver of C starvation – declining photosynthesis – actually drives increased allocation to storage carbohydrates during the early phases of stress.

Similar to an inability to utilize carbohydrates at the cellular level, phloem transport failure during drought is also likely to exacerbate C starvation. As stated by Sala *et al.*, this is a critical, but under-studied, question. It is likely that drought can reduce phloem transport via multiple mechanisms, such as lowering carbohydrate loading and unloading (including reduced sink activity), or by lowering phloem conductance by increasing sap viscosity (Chaves *et al.*, 2003; Hölttä *et al.*, 2009). Does phloem transport matter to drought survival? Some studies show that photosynthesis declines faster than assimilate transport during drought (Sung & Kreig, 1979; Fig. 1), but others indicate the opposite (citations in Sung & Kreig, 1979). These studies, however, do not achieve the limit of lethal drought stress and therefore the patterns could change abruptly before mortality (Fig. 1). For example, if photosynthesis fell to near zero (as occurs in piñon pine trees, McDowell *et al.*, 2008) or phloem transport suddenly ceased at −3.0 MPa (as suggested in Fig. 1), then the risk of C starvation would increase. Phloem transport failure could also facilitate drought-induced mortality if the movement of stored carbohydrates, nutrients, or metabolic signals is critical for survival (if storage within local organs is insufficient to

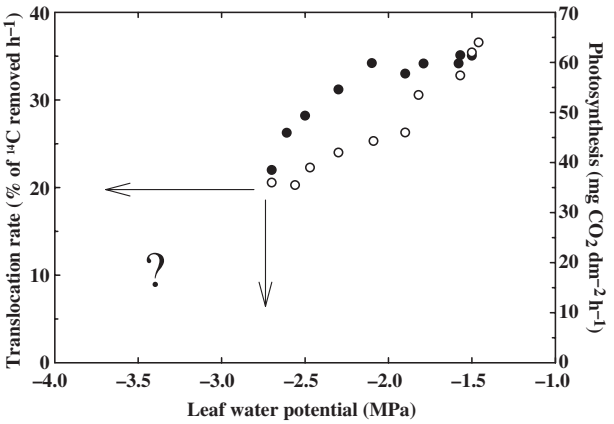

Fig. 1 Photosynthesis and ^{14}C loss from leaves (primarily translocation with a small amount lost to respiration, Hofstra, 1967) as a function of leaf water potential for sorghum plants. Data were reproduced from Fig. 3 in Sung & Kreig (1979). The arrows indicate that, while these data are tantalizing, they do not inform us regarding the responses near the lethal limits and thus may not be informative regarding mortality and survival mechanisms. Translocation, closed circles; photosynthesis, open circles.

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outlast drought), or if transport is needed to reduce foliar osmotic stress (Bartels & Sunkar, 2005). Relatively large carbohydrate reserves may have been stored before the late stages of drought (i.e. citations in Sala *et al.*), but the ability to extract them from storage and to transport them varies widely (Chaves *et al.*, 2003; and citations in Sala *et al.*). Finally, failure of phloem transport could be important if autophagy, or breakdown and recycling of cellular contents, is an important mechanism to mobilize resources to avoid mortality (Munne-Bosch & Alegre, 2004); thus, transport may be particularly critical during the final stages of survival. While phloem transport failure is unlikely to kill plants directly, it could play a critical role in promoting C starvation or other negative consequences that lead to mortality. Because phloem transport failure may have different impacts on sink and source organs, analyzing the sugar contents in different tissues up to the point of mortality will be critical in future experiments.

We applaud the goal of Sala and colleagues of furthering our understanding of vegetation mortality. A number of key questions have arisen from the current discussion. What fraction of stored carbohydrates is truly available to respiratory metabolism? What is the role of starch and other sugars in survival mechanisms, including phloem function? How do these carbohydrates vary at the whole plant level and across taxa? What is the interaction of hydraulics, metabolism and phloem transport? What is the global response of the above processes and their drought-dependence leading up to, and including, the point of mortality? We suggest that future research regarding mortality clearly distinguishes evidence from plants that actually die versus plants that are stressed but survive. The limited research that has been conducted in an appropriate manner to investigate mortality mechanisms (i.e. Marshall & Waring, 1985; Adams *et al.*, 2009) has yielded results consistent with C starvation, as formulated by both McDowell *et al.* (2008) and Sala *et al.*, but they do not necessarily 'prove' C starvation as the mechanism of mortality. Unfortunately, no studies have tested the C-starvation or hydraulic-failure hypotheses, in any of their forms, and sufficiently concluded that other mechanisms are not interacting or driving mortality. To improve our understanding of how plants die, new experiments explicitly designed to partition the different mechanisms need to be conducted.

Acknowledgements

This manuscript benefited from valuable discussions with many colleagues. NGM and SS were supported during the writing of this paper by the US Department of Energy, Office of Science (BER) and by LANL-LDRD.

Nate G. McDowell* and Sanna Sevanto

Earth and Environmental Sciences Division, Los Alamos National Laboratory, Los Alamos, NM, USA
(*Author for correspondence: tel +1 505 665 2909; email mcdowell@lanl.gov)

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Key words: carbon (C), drought, hydraulics, mortality, phloem, stomata.

A glimpse into the past of land plants and of their mycorrhizal affairs: from fossils to evo-devo

Arbuscular mycorrhizal (AM) fungi (AMF) have entered the mainstream of biology only in recent times, as a result of developments in DNA technologies and genomics, which are providing new tools to identify symbiont diversity, communication and development, and to reveal the contribution of each partner to the functioning of the symbiosis (Parniske, 2008; Bucher *et al.*, 2009). In this issue of *New Phytologist*, Bin Wang and colleagues (pp. 514–525) indirectly demonstrated the antiquity of the plant–AM fungal association. They elegantly used molecular tools, in an evo-devo manner, to show that this widespread association is probably homologous in all lineages of land plants (Embryophyta).

'Ironically, the mycorrhizal association is more ancient than the roots defining it (-rhiza), which arose within vascular plants only.'

Arbuscular mycorrhizas and other symbioses in land colonization

After surveying past and recent literature in this field, even an inattentive reader can quickly identify a few re-occurring assumptions on AM associations that are widely shared by the scientific community. One is that AMF improve plant nutrient uptake through the fine exploration of the rhizosphere and nearby soil by hyphae, whose network can extract nutrients that are otherwise not available to the plant. This has been soundly supported by many investigations, which have convincingly demonstrated the nutrient transfer and the underlying mechanisms (Bucher *et al.*, 2009). Equally popular is the statement that AMF, that is the Glomeromycota, have co-evolved with plants for at least the last 400 million yr, and that this association allowed the conquest of dry terrestrial ecosystems by Embryophyta (Selosse & Le Tacon, 1998; Wang & Qiu, 2006). Recent reanalyses of early enigmatic terrestrial fossils suggest that the first macroscopic land colonizers were fungal–algal associations, such as *Prototaxites* (Selosse, 2002) or *Spongiophyton* (Taylor *et al.*, 2004). Indeed, adding an alga (which is able to exploit light and CO_2 from the environment) to a fungus (able to exploit the soil substratum) makes a perfect adaptation to living at the soil–atmosphere interface, the landmark of land plants. Evidence supporting the antiquity of plant–AMF associations have so far been limited to molecular clock analyses using DNA sequences (Simon *et al.*, 1993), and to fossils analyzed in the original paper by Kidston & Lang (1921) and in the overlooked paper by Boullard & Lemoigne (1971), both based on the 400-million-yr-old Rhynie Chert deposits. However, the Rhynie Chert deposits are more recent than the earliest land plants (Wellman *et al.*, 2003), and none of the previous indirect evidence provides insights into the functioning of the association.

Interestingly, AMF colonize extant basal plant lineages, that is, some of the paraphyletic 'bryophytes' (liverworts = hepatics and hornworts; Russell & Bulman, 2005; Ligrone *et al.*, 2007), as well as the Lycophyta (Winther & Friedman, 2008) and ferns (Boullard, 1979). This stimulated comments on the association of plant ancestors with AMF (Kottke & Nebel, 2005; Selosse, 2005; Leake *et al.*, 2008) and on the capacity of AMF to colonize plant tissues irrespectively of the gametophytic or sporophytic status (Bonfante & Genre, 2008). Arbuscular mycorrhizal fungi can, in fact, colonize haploid gametophytic tissues with different degrees of success: while mosses such as *Physcomitrella patens*, a model organism for basal plants, do not seem to be susceptible to colonization by AMF, many liverworts and hornworts gametophytes are successfully colonized in field (Ligrone *et al.*, 2007) and *in vitro* (Fonseca & Berbara, 2008) conditions, as are some clubmosses and ferns (Boullard, 1979). In ferns, the set-up of an *in vitro* system allowed one of us to demonstrate that only the diploid sporophyte of *Pteris vittata* shows the classical mycorrhizal phenotype (Fig. 1), confirming that the presence of AMF in the fern gametophyte is more erratic (as first noted by Boullard, 1979).

The *sym* genes and their evolution

Wang and colleagues provide the first demonstration that the molecular mechanisms of the signalling transduction pathway that control the AM symbiosis are also present in basal land plants, and that AMF colonization is thus a homologous process in all Embryophyta lineages, both in gametophytes and in sporophytes. They investigated the well-known *sym* genes, which have been instrumental in identifying the first steps of symbiosis establishment. Seven of these genes have been identified in *Lotus japonicus* and four in *Medicago truncatula* (Parniske, 2008) and they were demonstrated to be essential in the signalling transduction pathway leading to both AMX and rhizobial symbiosis. It has already been shown that some of these genes were conserved during plant evolution: their presence in monocots,

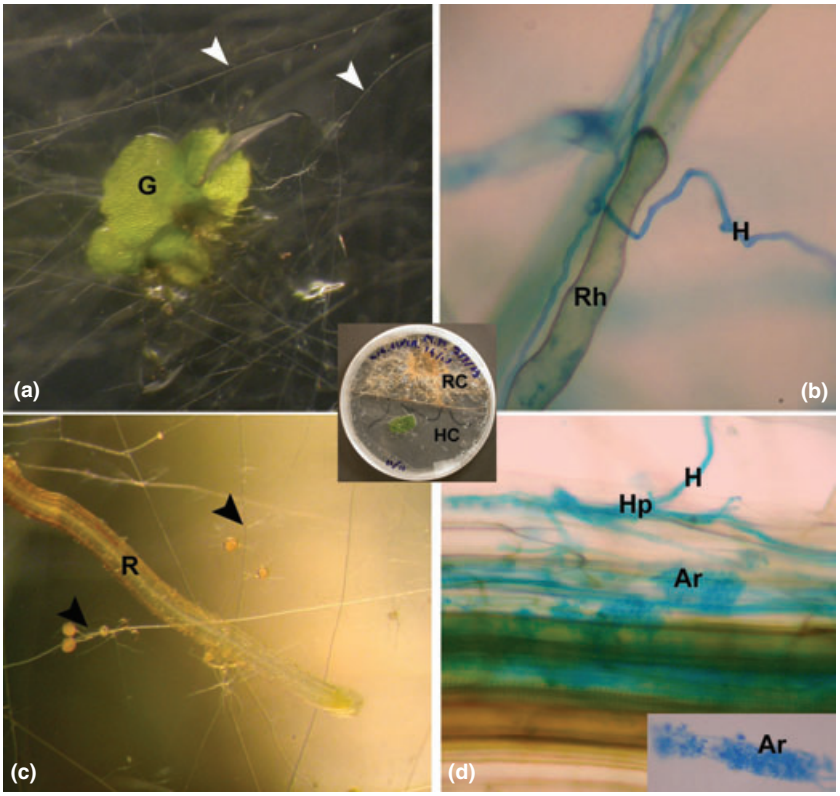

Fig. 1 Arbuscular mycorrhizal (AM) associations in the fern *Pteris vittata* are restricted to the diploid sporophyte (M. Novero, *et al.*, unpublished). (a) Gametophytes of *P. vittata*, obtained according to Trotta *et al.* (2007), were transferred to septate Petri dishes, in compartment HC, where *Glomus intraradices* proliferates (arrows) fed by the transformed carrot roots from compartment RC. (b) Hyphae (H) contacted the gametophytic rhizoids (Rh), but colonization events were never observed. (c) Sporophytes (R) developed in the Petri dish after 30 d, while *G. intraradices* produced spores and branched hyphae (arrowheads). (d) The AM hyphae contacted sporophytic root cells, producing hyphopodia (Hp), intracellular hyphae and, lastly, arbuscules (Ar).

such as rice (*Oryza sativa*), suggests that the associated functions predated the monocots–eudicots divergence (Chen *et al.*, 2009). Starting from this level of knowledge, Wang and colleagues tested the hypothesis of whether orthologs of the *sym* genes were already present in the genome of the basal plants, which spend most of their life cycle in the form of a haploid gametophyte.

They focused on three *sym* genes, which, according to the *Medicago* nomenclature, are described as follows: *DMI1*, coding for a cation channel located in the nuclear envelope; *DMI3*, coding for a calcium/calmodulin-dependent kinase (CCaMK), which acts downstream of the calcium spiking during the perception of the nodulation factor in *Rhizobium* symbiosis; and lastly *IPD3*, coding for a protein that directly interacts with, and is phosphorylated by, CCaMK. Homologs of these genes were successfully isolated from a long list of plants from the major Embryophyta lineages: liverworts, mosses, hornworts, lycophytes, ferns, gymnoperms and, of course, angiosperms. Phylogenetic analyses showed that these genes had been vertically inherited during the evolution of land plants, as the gene phylogenies largely agreed with the Embryophyte phylogeny recently reconstructed from a large body of molecular data (Qiu *et al.*, 2006). To demonstrate the function of such genes, complementation experiments were performed to demonstrate that the *DMI3* genes from liverworts and hornworts could rescue *M. truncatula* dmi3 mutants, which are affected in their mycorrhizal capacities. The transformed *Medicago* roots showed, in fact, some of the expected mycorrhizal features, such as intraradical hyphae, vesicles and sometimes also arbuscules. Moreover, yeast two-hybrid assays also confirmed that the DMI3 proteins from liverworts and hornworts interact with the downstream-acting IPD3 protein in *Medicago*.

These results convincingly suggest that the *sym* genes were already present in ancestral plants and that their function was conserved during evolution. They were maintained during changes in plant life cycles (i.e. the evolution of a gametophyte-dominated or a sporophyte-dominated cycle; Langdale & Harrison, 2008), leading to the concept that root colonization is homologous to that of rootless liverworts and hornworts' thallus. The absence of amplifiable *sym* genes in Charales, green algae that are possible sister taxa to Embryophyta (Becker &

Marin, 2009), but the presence in these algae and land plants of a calcium-dependent protein kinase (CDPK; Wang *et al.*) add original information: on the one hand, the discovery points to an ancient innovation that was acquired during terrestrialization; on the other hand, the phylogenetic analysis of *CDPK* and *DMI3* strongly suggests that the latter originated by duplication, with gain of a new function. Freshwater green algal taxa, like the Coleochaetales and the Zygnematales, whose sister position to Embryophyta is still hotly debated (Becker & Marin, 2009), now deserve investigation. Last, the findings of *sym* genes in taxa that do not associate with AMF, such as mosses (*Physcomitrella*) and *Arabidopsis thaliana*, suggest that the corresponding protein may have roles unrelated to the AM symbiosis in some Embryophyta, and this deserves further research.

A window on early land plants?

Of the many questions raised by the work of Wang and colleagues, the first concerns the symbiotic status of the ancestral land plants. Is AM symbiosis a plesiomorphic feature? Indeed, the presence of the *sym* genes in non-AM plants opens the possibility of a repeated recruitment of the same transduction machinery during independent emergence of the AM symbiosis. For example, the repeated emergence of C_4 photosynthesis in angiosperms entailed the parallel evolution of the gene for the key enzyme, the phosphoenolpyruvate carboxylase, to similar, or even identical, amino acid sequences (Christin *et al.*, 2007). Interestingly, in non-AM mosses, except for *Takakia* that associates with AMF, the *sym* genes are undergoing a divergent, positive selection (as also shown by a long branch for non-*Takakia* mosses on Fig. 1 of Wang *et al.*). This pattern suggests that the situation of *sym* genes in non-AM mosses is a derived feature and that the role of *sym* genes in the AM symbiosis is driving their evolution in Embryophyta. Analysis of the sequences and roles of *sym* genes in sister taxa of Embryophyta, if any can be found, would provide additional evidence for this.

Recently, the diversity of morphological features of AM–liverwort associations, and results showing that fungi associated with the extant liverworts mostly belong to Glomerales group A, a relatively recent AM fungal taxon (Russell & Bulman, 2005), pushed Selosse (2005) to consider the possibility of a 'host shifting' in extant bryophytes. Under this hypothesis, independently of the fact that the Embryophyta ancestor was probably mycorrhizal (Selosse & Le Tacon, 1998), several AMF could have recently colonized some basal Embryophyta lineages, so that extant AM symbioses could be secondary reversion or convergence in liverworts and/or hornworts (Selosse, 2005). Further investigations by Ligrone *et al.* (2007), on a larger number of liverworts from different geographic origins, confirmed the dominant presence of Glomerales from group A, although a Diversisporales-related sequence was also detected. These data again suggested that extant liverworts are more frequently associated with recent AM fungal taxa, in line with the previous hypothesis. However, unpublished results by M. Naumann, A. Desirò and P. Bonfante on *Conocephalum*, a common liverwort species, identified sequences related to diverse and more ancient taxa, such as Diversisporales and Archeosporales. Interestingly, these results were obtained by using long primers for the small subunit (SSU) region amplification recently set up by Lee *et al.* (2008), perhaps overcoming the technical limitations of previous primer sets.

In conclusion, the phylogenetic analysis by Wang and colleagues, demonstrating the presence of the *sym* genes in basal plants and the presence in liverworts of ancient VA fungal taxa, strongly support the concept that AM symbioses are likely to be homologous, that is, a trait derived from the common ancestor of all Embryophyta (Selosse & Le Tacon, 1998; Kottke & Nebel, 2005; Wang & Qiu, 2006). Thus, (1) AM symbiosis should now join the synapomorphies (i.e. traits shared by two or more taxa and their last common ancestor) of Embryophyta in textbooks and (2) as a result, extant non-mycorrhizal lineages did secondarily (and independently) lose AM symbiosis during Embryophyta evolution. Ironically, the mycorrhizal association is more ancient than the roots defining it (-rhiza), which arose only within vascular plants. However, this does not necessarily entail that the morphology of the plants' common ancestor and its AM association were close to that of extant liverworts and hornworts (Selosse, 2005; Bonfante & Genre, 2008). In this respect, one may hope to unravel well-preserved fossils and rich *Lagerstätten*, older than the 400-million-yr-old Rhynie Chert where Embryophyta and AM symbioses had already diversified (Boullard & Lemoigne, 1971; Krings *et al.*, 2007).

Acknowledgements

We thank M. Novero, A. Trotta, M. Naumann and A. Desirò for sharing unpublished data, and Professor Yin-Long Qiu for critical reading of this commentary.

Paola Bonfante[1] and Marc-André Selosse[2]*

[1]Dipartimento di Biologia Vegetale dell'Università di Torino and Istituto per la Protezione delle Piante del CNR, Sezione di Torino, Viale Mattioli, 25, 10125 Torino, Italy;
[2]Centre d'Ecologie Fonctionnelle et Evolutive, CNRS, UMR 5175, Equipe Interactions Biotiques, 1919 Route de Mende, 34 293 Montpellier cedex 5, France
(*Author for correspondence: tel 33 (0)4 67 61 32 30; email ma.selosse@wanadoo.fr)

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Key words: arbuscular mycorrhiza, land colonization, molecular tools, plant ancestors, signal transduction pathways, symbiosis.

Letters

An efficient procedure for normalizing ionomics data for *Arabidopsis thaliana*

We propose an efficient procedure for normalizing *Arabidopsis thaliana* ionomics data. The ionome is the mineral nutrient and trace element composition of an organism, which includes elements nonessential for plant growth (Salt *et al.*, 2008; Baxter, 2009). The ionome can be considered as the inorganic subset of the metabolome at a given moment in space and time, and it is dependent on evolutionary, genetic, developmental and environmental factors. The ionomes of several plant species (e.g. *A. thaliana*, *Brassica*, *Oryza sativa* (rice) and *Lotus*) are currently being characterized (Lahner *et al.*, 2003; Broadley *et al.*, 2008; Salt *et al.*, 2008; Chen *et al.*, 2009; Hammond *et al.*, 2009; White *et al.*, 2010), as is the ionome of baker's yeast

(*Saccharomyces cerevisiae*; Eide *et al.*, 2005; Danku *et al.*, 2009). The most comprehensive ionomics data by far are for *A. thaliana* (Salt *et al.*, 2008). Currently, these data comprise 128 238 unique samples from 10 386 genotypes/lines/accessions, and include 17 785 fast-neutron (FN) mutagenized plants, 24 685 T-DNA mutagenized plants (representing 1941 unique genes), 14 258 ethylmethane sulfonate (EMS) mutagenized plants and 32 280 wild-type plants, including 522 different accessions and inbred lines (all data were obtained from a database download from http://www.ionomicshub.org on September 23 2009). Several genetic correlates of mutant mineral phenotypes have been identified. For example, among FN mutants, a major calcium (Ca) phenotype was recently identified for a gene (enhanced suberin biosynthesis 1, *ESB1*) that controls the radial apoplastic transport of Ca to the root stele (Baxter *et al.*, 2009).

An alternative to screening induced mutations for altered ionomic phenotypes is to exploit the reservoir of natural variation in *A. thaliana* (Nordborg *et al.*, 2005). Currently, the ionomes of recombinant inbred progeny of six mapping populations and > 360 natural accessions have been reported. Correlating variation in genotype with phenotype among natural accessions – after correcting for population structure – has identified alleles that are under selection and are of putative adaptive significance. For example, a low shoot molybdenum (Mo) concentration among 92 *A. thaliana* accessions has been linked to a naturally occurring deletion in the promoter of a mitochondrion-localized transporter (molybdenum transporter 1, *MOT1*; Baxter *et al.*, 2008a). Furthermore, higher shoot sodium (Na) accumulation in two accessions from the Tossa del Mar (Ts-1) and Tsu (Tsu-1) sites, on the coastal regions of Spain and Japan, respectively, has been linked to a novel At*HKT1* allele (Rus *et al.*, 2006). Natural genetic resources are still relatively underexploited, primarily because natural phenotypic variation associates with genotypic variation at multiple loci.

A. thaliana ionomics data are being generated by high-throughput phenotyping. Data are managed using the Purdue Ionomics Information Management System (PiiMS) and are publicly available at http://www.ionomicshub.org for viewing, download and re-analysis. The workflow is described in detail elsewhere (Lahner *et al.*, 2003; Baxter

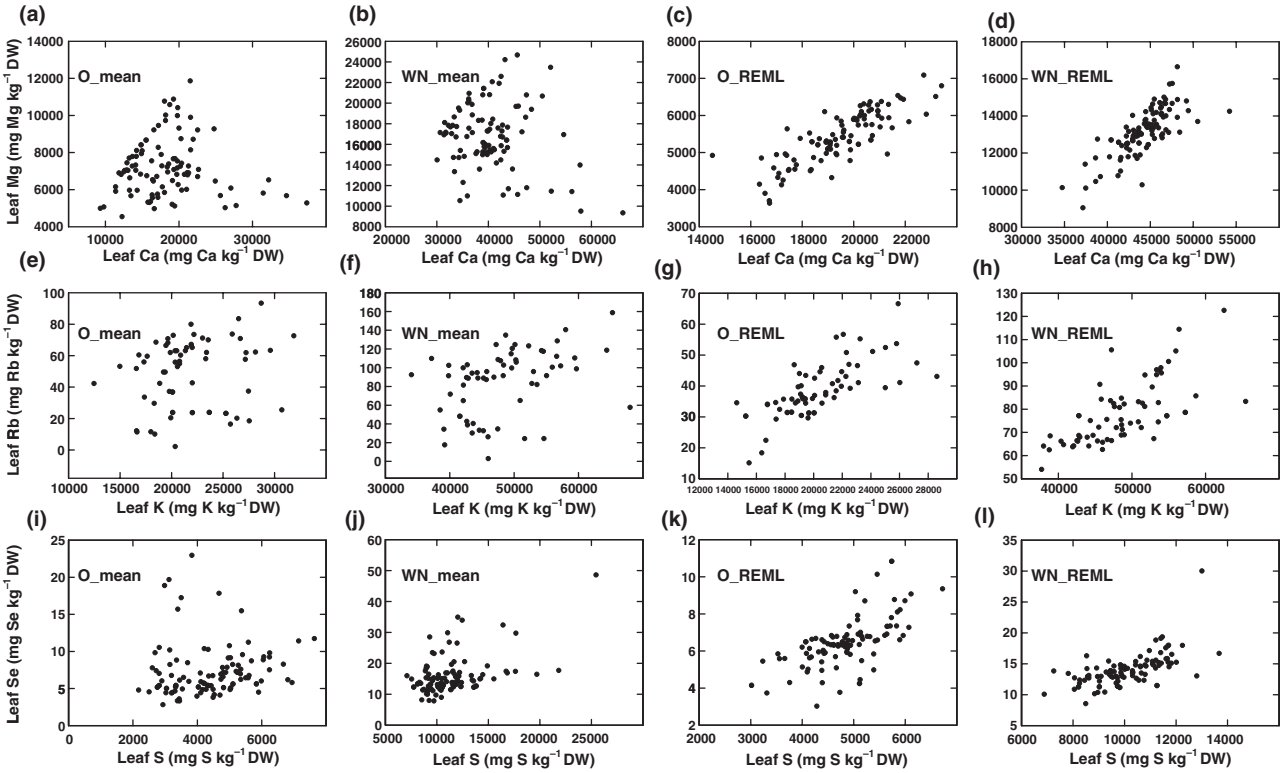

Fig. 1 Relationship in leaf mineral concentration between three element-pairs among natural accessions of *Arabidopsis thaliana*. (a–d) Calcium (Ca) and magnesium (Mg) concentrations (n = 96); (e–h) potassium (K) and rubidium (Rb) concentrations (n = 63); (i–l) sulfur (S) and selenium (Se) concentrations (n = 96). Accessions are as defined in Nordborg *et al.* (2005) and data were downloaded from http://www.ionomicshub.org/arabidopsis/piims/showIndex.action (May 2009). Panels labelled 'O_...' refer to observed data values (units, μg l^{-1}); panels labelled 'WN_...' refer to weight-normalized values (units, mg kg^{-1} dry weight). Panels labelled '..._mean' are arithmetic means of accessions; panels labelled '..._REML' are accession means estimated using a residual maximum-likelihood procedure. Correlation coefficients are provided in Table 1.

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Table 1 Correlation coefficients between leaf mineral concentrations of element-pairs, based on arithmetic means, or residual maximum likelihood (REML)-estimated means, for 96 accessions of *Arabidopsis thaliana*, grown in 210 trays with Col-0 common to all trays (see Fig. 1 for related scatter plots)

Element correlation	Observed values		Weight normalized values	
	R (means)[a]	*R* (REML)	*R* (means)	*R* (REML)
Ca : Mg	0.005^{ns}	0.818	0.175^{ns}	0.769
K : Rb	0.202^{ns}	0.705	0.384	0.701
S : Se	0.078^{ns}	0.650	0.476	0.635

[a]Correlation coefficient. All are significant at $P < 0.0001$, except those marked 'ns', which are $P > 0.05$.
Ca, calcium; K, potassium; Mg, magnesium; Rb, rubidium; S, sulfur; Se, selenium.

et al., 2007). Briefly, genotypes/lines/accessions are sown in 'trays' comprising 70–108 individual units and with Col-0 included as a reference accession in each tray. There are a minimum of six individual replicate plants per genotype/line/accession within a tray. The sowing pattern is varied across trays to reduce positional effects. Seeds are sown onto moist soil, stratified at 4°C for 48–72 h and grown for 36–40 d in a climate-controlled room at 19–24°C with 10 h of photosynthetically active light at 80–100 μmol m^{-2} s^{-1}. Two or three leaves (with a total fresh weight of 1–5 mg) are sampled for subsequent mineral analysis using inductively coupled plasma mass spectometry (ICP-MS). The leaf mineral composition for each element is determined using a per-tray global weight-normalization procedure, based on the leaf content of all elements (Lahner *et al.*, 2003). This allows for accurate comparison of genotypes/lines/accessions within each tray. There are currently > 1500 'trays' of *A. thaliana* data in the public domain that have been generated continuously since October 2001.

Inter-tray comparison of genotypes/lines/accessions is nontrivial. Despite the maintenance of standard conditions during the *A. thaliana* ionomics workflow, plants inevitably experience slight variation in environmental conditions (temperature, watering, etc.) between experiments. Furthermore, certain environmental conditions (e.g. nutrient supply) are manipulated deliberately for experimental reasons. For data analysis, because there is at least one accession (Col-0) common to all experiments, we can consider the workflow as delivering a set of related experiments with synonymous 'experiment' and 'tray' terms. The workflow can also be considered as an incomplete block-design experiment, with 'accession' representing the fixed – i.e. treatment – factor. All random factors, including environmental and technical (e.g. analytical) variation, integrate at the level of 'tray'. Residual maximum likelihood (REML) procedures (Patterson & Thompson, 1971; Robinson, 1987; Welham & Thompson, 1997) can provide reliable estimates of treatment means and can recover information on variance structures, from incomplete block-designs and from sets of related experiments with unknown error sources (e.g. Broadley *et al.*, 2001, 2008; Watanabe *et al.*, 2007). Residual maximum likelihood procedures can also be used to determine the relative contributions of genotypic and environmental sources of variation acting on the phenotype.

Here, we have tested the hypothesis that REML-estimated means are a reliable and efficient method for normalizing *A. thaliana* ionomics data across multiple trays. We tested this hypothesis using a core set of 96 natural accessions of *A. thaliana*, representing most of the common allelic variants within the species (Nordborg *et al.*, 2005), grown across 210 trays (http://www.ionomicshub.org/arabidopsis/piims/showIndex.action; downloaded May 2009). Data were identified using the accession name. Observed (solution concentration in μg l^{-1}, measured using ICP-MS) and weight-normalized (mg kg^{-1} dry weight) data were downloaded for cells in each tray containing the accession, and data for cells containing Col-0 in the same tray were downloaded in a similar manner. Data were analysed separately for each element. For the REML procedure, we used an additive linear mixed model with 'accession' defining the fixed-treatment effect and 'tray' defining the sum of random effects. This approach partitions the total variation in leaf mineral concentration into components of an addi-

Table 2 Variance components analyses of leaf mineral concentration from the residual maximum likelihood (REML) procedure using the random model of (Accession + (Accession/Tray)) for 96 accessions of *Arabidopsis thaliana*, grown in 210 trays with Col-0 common to all trays

Model term	Proportion of variation in leaf mineral nutrient concentration explained by model term (%)					
	Ca	Mg	K	Rb[a]	S	Se
Accession	0.87	0.97	4.92	1.39	2.92	0.51
Tray	85.21	88.73	53.06	87.33	63.03	90.10
Accession × Tray	0.28	0.53	0.17	−0.21	1.15	0.15
Residual term (plant-to-plant variation)	13.65	9.77	41.85	11.49	32.90	9.24
n	12 175	12 175	11 826	3450	5593	12 175

[a]Negative variance components values can arise during model fitting; here, the Accession × Tray term is effectively zero.
Ca, calcium; K, potassium; Mg, magnesium; Rb, rubidium; S, sulfur; Se, selenium.

tive model and provides estimated means for the 'accession' fixed factor from the model. Thus, variation in leaf mineral concentration caused by the genotype is assigned to the 'accession' term. Variation in leaf mineral concentration as a result of environmental effects, or plant-to-plant effects, is assigned to the 'tray' and 'residual' terms, respectively. The approach is analogous to adjusting a treatment mean in a designed experiment by removing a blocking term. By comparing the proportion of variation in leaf mineral concentration explained by the model terms, we were therefore able to determine the scale of the genotype effect compared with the tray-to-tray and plant-to-plant variability. Such techniques have previously been used to extract evolutionary information from other unstructured data sets (Watanabe *et al.*, 2007, and references therein). Statistical analyses were conducted using GENSTAT (Release 12.1.0.3338; VSN International Ltd, Hemel Hempstead, UK).

As the aim of the ionomics workflow is to determine 'relative' leaf mineral composition with precision, and not 'absolute' mineral concentrations, we tested the effectiveness of REML by exploring the relationships between the leaf concentrations of three pairs of elements (Ca and magnesium (Mg); potassium (K) and rubidium (Rb); and sulfur (S) and selenium (Se)). We expected these three element-pairs to correlate in absolute terms, based on previous studies (Broadley *et al.*, 2004; Watanabe *et al.*, 2007; White *et al.*, 2007). The correlations between all three element-pairs improved substantially when data were REML-normalized across trays (Fig. 1, Table 1). This improvement was consistent for observed leaf concentrations and for weight-normalized leaf concentrations. For example, mean weight-normalized leaf Ca and Mg concentrations do not correlate across 96 accessions ($R = 0.17$, $P > 0.05$; Fig. 1d). Following REML normalization, leaf Ca and Mg concentrations are highly correlated ($R = 0.82$ and 0.77; Fig. 1a,b), as are leaf K and Rb ($R = 0.71$ and 0.70; Fig. 1e,f) and leaf S and Se ($R = 0.65$ and 0.64; Fig. 1i,j). Additional correlation structures between elements in the ionomics database (i.e. 'mineral signatures') remain to be explored.

A valid criticism is that the REML model can only achieve successful normalization of leaf mineral concentration on a per-accession basis if nongenotypic effects (represented in this analysis by 'tray') are reasonably uniform across all genotypes/lines/accessions, and if these effects are additive. If the environment induces significant Genotype × Environment interactions, or if nongenotypic effects are nonadditive, then the process is compromised. To explore this aspect, we examined the proportion of variation in leaf mineral concentration explained, by each of the model terms, using a (Accession + (Accession/Tray)) random model (Table 2). Accession effects, in general, are small, compared with plant-to-plant and tray-to-tray variation, which dominate the analysis. Furthermore, the proportion of variation in leaf mineral concentration explained by the Accession × Tray interaction terms is relatively small for each element, compared with Accession effects alone. Given this relatively small contribution of the Accession × Tray interaction term to explain variation in leaf mineral concentration in *A. thaliana*, the REML procedure appears to be appropriate, although clearly one must proceed with any normalization step with caution. Despite being globally small, the interaction term is where ionomics can undoubtedly capture some of the most interesting aspects of plant biology. More detailed variance components analysis will guide future experimental designs to explore genotype-specific responses to environment.

From these observations, we conclude that REML normalization is an efficient procedure for estimating leaf mineral concentrations from the *A. thaliana* ionomics database. Such normalization should facilitate the more efficient exploration of the large ionomics data sets currently being developed, because these data sets are composed of many hundreds of experiments extending across multiple years. Comparison of the ionome of different genotypes across the multiple experiments will enhance the ability to identify informative mutants and natural variants, as well as allow the identification of classes of ionomic profiles with common underlying physiological foundations (Baxter *et al.*, 2008b).

Acknowledgements

BBSRC Agri-Food IPA, BB-G013969-1 (MRB and JPH); the Scottish Government Rural and Environment Research and Analysis Directorate (PJW); US National Science Foundation (IOB 0419695) and the US National Institutes of Health (R01 GM078536) (DES).

M. R. Broadley[1]*, J. P. Hammond[2], P. J. White[3] and D. E. Salt[4]

[1]Plant and Crop Sciences Division, University of Nottingham, Sutton Bonington, Loughborough, LE12 5RD, UK; [2]Warwick-HRI, University of Warwick, Wellesbourne, CV35 9EF, UK; [3]Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK; [4]Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN, 47907, USA
(*Author for correspondence: tel +44 (0)115 9516382; email martin.broadley@nottingham.ac.uk)

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Key words: Arabidopsis, biofortification, calcium (Ca), ionome, magnesium (Mg), mineral nutrition, natural variation, potassium (K).

Physiological mechanisms of drought-induced tree mortality are far from being resolved

Introduction

The recent worldwide increase of drought-induced tree mortality exacerbated by climate change (see review by Allen *et al.*, 2009 and references therein) has triggered strong interest in revisiting the exact mechanisms leading to mortality (McDowell *et al.*, 2008). In addition to biotic factors such as pathogens and insects, which amplify the negative effects of drought or are amplified by elevated temperatures and drought (Desprez-Loustau *et al.*, 2006; Rouault *et al.*, 2006), two physiological mechanisms have been advanced in the literature and recently formalized by McDowell *et al.* (2008) within a hydraulic framework: hydraulic failure and carbon (C) starvation. Hydraulic failure occurs when drought intensity is sufficient to push a plant past its threshold for irreversible desiccation. The C starvation hypothesis has been invoked in the literature for decades (Parker & Patton, 1975) and predicts that reduced C assimilation via photosynthesis as a result of drought-induced stomatal closure – particularly in isohydric species that close stomata to maintain high (i.e. less negative) xylem water potentials (McDowell *et al.*, 2008) – leads to an imbalance between C availability and C loss as a result of metabolic demands (e.g. growth and maintenance). Over time, if drought persists, such negative C balance can lead to an exhaustion of C reserves and, ultimately, to C starvation and death.

Although mortality resulting from hydraulic failure is extremely difficult to demonstrate in nature, lower resistance to cavitation (Rice *et al.*, 2004; Pratt *et al.*, 2008) and lethal leaf dehydration as a result of hydraulic failure (Kursar *et al.*, 2009) have been related to increased mortality in the field, rendering hydraulic failure a very plausible mechanism for observed tree mortality under severe, acute drought. Here, we focus on the carbon starvation hypothesis (hereafter CSH) as proposed by McDowell *et al.* (2008). The specific prediction of the CSH is that drought-

induced stomatal closure to prevent irreversible dessication leads to near-zero C assimilation and that 'continued demand for carbohydrates to maintain metabolism will deplete carbohydrate reserves, leading eventually to starvation or an inability to fend off attack from biotic agents, whichever comes first' (McDowell *et al.*, 2008). Our criticism focuses on the C starvation prediction component (i.e. the CSH as stated in Fig. 3 of McDowell *et al.*, 2008) and is based on lack of direct evidence that trees exposed to drought, but not suffering mortality from other mechanisms, end up dying of C starvation as a result of an exhaustion of stored reserves; and that the CSH rests on untested assumptions and ignores other possible mechanisms. While conceptually very appealing and logical, we argue that the hydraulic framework as proposed by McDowell *et al.* (2008) is problematic and deserves further evaluation. Because of this, we believe it is dangerous to invoke the CSH as the most likely causal explanation for drought-induced tree mortality in the absence of hydraulic failure or biotic agents before the direct evidence is at hand. Invocation of the CSH (Adams *et al.*, 2009; McDowell *et al.*, 2010; but see Sala, 2009 and Leuzinger *et al.*, 2009) may lead to premature acceptance and detract from critical tests of key assumptions, exploration of contingent hypotheses and, ultimately, the advancement of the field.

Terms and definitions

Our discussion requires the distinction between four interrelated terms: C depletion, C limitation, C-reserve exhaustion, and C starvation (Fig. 1). Let us assume for now that drought limits photosynthesis (because of stomatal closure) and C demand for growth (because of turgor-limited cell expansion; Ishii *et al.*, 2008). Depending on the relative decrease of photosynthesis and C demand, C balance may be zero, positive or negative. If C balance is zero or positive, storage C pools will remain stable or increase, respectively. If the C balance is negative, stored C will make up for the difference and the concentration of stored C will decrease, leading to a depletion of stored C (C depletion or decrease of stored C). If drought does not last long enough such that storage pools are sufficiently large to meet sink demand, C does not become limiting and plants can survive. However, if drought is long enough and reserve C pools cannot meet C demand, then plants are C-limited and some sink activity may be impaired (C limitation). If drought perseveres, C limitation will eventually lead to a complete depletion of stored C pools (C-reserve exhaustion) which, in turn will lead to insufficient C supply to fuel cell metabolism (true C starvation at the cellular level) and to mortality. The CSH follows this logic. However, we argue that drought-induced

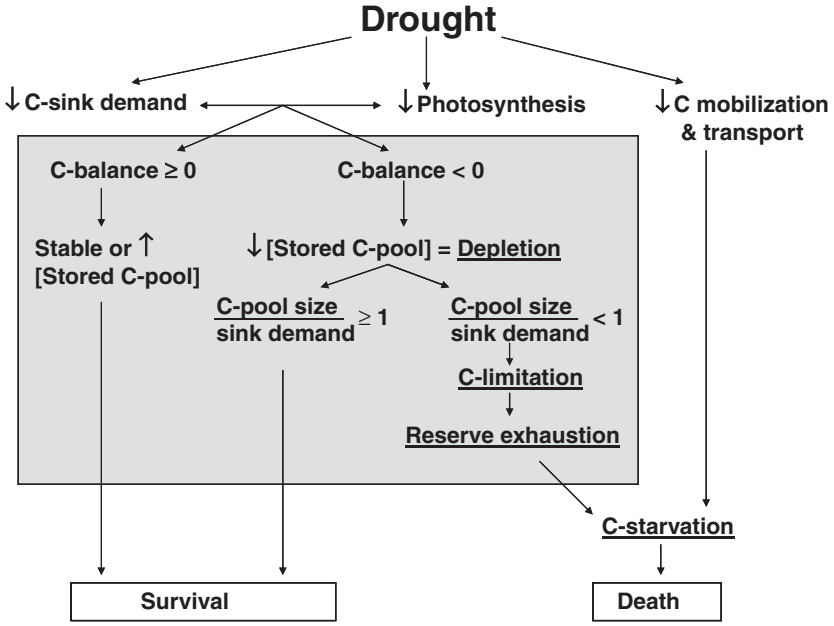

Fig. 1 Effects of drought on carbon (C) assimilation, demand and transport, and possible consequences for plant survival. The grey area depicts possible effects of drought on whole-plant C balance and on the concentration of stored C pools, assuming no constraints on C mobilization and transport. In this case, plant survival is likely when C balance remains positive. It is also likely even under negative C balance, causing a depletion of stored C pools (decrease in concentration), if such C pools remain large enough to sustain overall sink demand. If the C pool becomes insufficient to sustain overall sink demand, plants become C limited. Under prolonged drought, C reserves may eventually be exhausted, leading to C starvation and death. However, negative effects of drought on C mobilization and transport could lead to insufficient C delivery to cells, which also leads to insufficient C supply for cell metabolism (i.e. C starvation at the cellular level) and subsequent death, regardless of the effects of drought on whole-plant C storage.

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mortality in the absence of hydraulic failure or biotic agents does not necessarily have to be preceded by this sequence of events (Fig. 1). That is, actual cellular C starvation may occur even when the C-reserve pool is not exhausted. From a physiological perspective, this distinction is critical because it involves fundamentally different mechanisms at the whole-plant level, with important implications for physiology and ecology (see later discussion).

Stored C is defined here as C accumulated in any organ or tissue during periods of C surplus (net assimilation > C demand), which can later be retrieved and transported to sinks where C demand exceeds available C (Chapin *et al.*, 1990). Most nonstructural organic compounds in plants can be recycled and serve as C sources for plants (Chapin *et al.*, 1990). However, nonstructural polysaccharides (starch and fructans) and neutral lipids (triacylglycerols) are exclusively synthesized for storage and are the most common forms of C storage. Low molecular sugars and sugar alcohols may also be used as C sources but their primary function is osmotic adjustment (Quick *et al.*, 1992; Arndt *et al.*, 2008). Therefore, water stress could theoretically lead to C-reserve exhaustion without a complete depletion of low-molecular-weight C compounds if these are used for osmotic adjustment (Arndt *et al.*, 2008).

Lack of direct evidence for C starvation preceding drought-induced mortality

To date, direct evidence to support the CSH is lacking: an actual exhaustion of stored C reserves preceding drought-induced mortality has never been shown. Although studies may be compatible with the CSH (see McDowell *et al.*, 2008), they do not provide the direct evidence necessary to rule out additional explanations. Furthermore, and consistent with Fig. 1, the concentration of mobile C pools in woody plants has been shown to remain stable, increase or decrease in response to water stress intensity and duration. This variability and the lack of direct evidence for the CSH suggest that acceptance of this hypothesis as a generalized mechanism to explain drought-induced mortality in trees in the absence of hydraulic failure and/or insect/pathogen attack may be premature.

Decreases of root starch concentration (i.e. C depletion) in seedlings subjected to drought (either by withholding water or by exposing roots to dry air before transplanting) has been shown in *Quercus velutina* (Parker & Patton, 1975), *Pinus taeda* (Green *et al.*, 1994) and *P. nigra* (Guehl *et al.*, 1993; Girard *et al.*, 1997). Guehl *et al.* (1993) noted that starch concentration in roots of *P. nigra* seedlings that survived water stress preconditioning and transplanting decreased with predawn water potential. They suggested C starvation as a mechanism for mortality based on severely depleted (although not zero) starch concentrations estimated at the water potential inducing 100% mortality. However, their results also suggest a parallel impairment of hydraulic function and highlight that the distinction of hydraulic failure from C starvation as the cause of mortality may be difficult. This interpretation is corroborated by the results by Girard *et al.* (1997) with the same species, where hydraulic failure was ruled out as a cause of mortality, and starch concentration in roots, while reduced, remained high even in seedlings that died.

Consistent with the CSH, Arndt *et al.* (2008) showed an almost complete exhaustion of starch in wood of young (10-yr-old) *Eucalyptus* under seasonal drought stress. The missing link is that C-reserve exhaustion was apparently not associated with tree mortality. Starch depletion in mature trees in response to water stress has also been reported in a few other cases. For instance, in mature oaks, drought-induced decreases of starch at the base of the trunk were related to subsequent extensive branch mortality (Bréda *et al.*, 2006). However, residual starch concentrations before mortality were far from exhaustion. Sayer & Haywood (2006) also concluded that root starch depletion occurred in mature *Pinus palustris* exposed to three consecutive years of prolonged drought. However, a close examination of their data suggests that the reported gradual depletion of starch under prolonged water stress is, at best, equivocal.

Although common, C depletion in woody plants in response to water stress is not ubiquitous. For instance, Villar-Salvador *et al.* (2004) reported no significant change in starch concentration in drought-hardened seedlings of *Quercus ilex* relative to well-watered seedlings. Moreover, starch and other C-reserve concentrations in woody plants have been shown to increase (rather than decrease) in response to decreases in water availability. This is the case in juvenile (Latt *et al.*, 2001) and mature (Würth *et al.*, 2005) tropical trees exposed to seasonal drought, in *Pinus ponderosa* growing in drier vs moister habitats (Sala & Hoch, 2009), and in seedlings of *Pinus palustris* (Runion *et al.*, 1999) and *Casuarina equisetifolia* (Sánchez-Rodríguez *et al.*, 1999) exposed to experimental drought. Consistently, experimental irrigation led to reserve-C depletion of adult trees and shrubs relative to nonirrigated trees (Tissue & Wright, 1995; Ludovici *et al.*, 2002). Carbon-reserve accumulation has also been reported in Mediterranean species subjected to extreme drought (Körner, 2003), and with increases in tree height associated with increasing water transport constraints (Sala & Hoch, 2009). Note that according to McDowell *et al.* (2008) isohydric species are more prone to C starvation as a result of early stomatal closure. Yet, increases in C-reserve concentrations with drought apparently also occur in isohydric species (e.g. pines).

This diversity of responses suggests that the relative sensitivity of sources and sinks to water stress varies depending on the species, the ontogenetic stage (seedlings vs adults) and the degree of water stress, and highlights that

the mechanisms for drought-induced mortality in trees are far from being resolved. Clearly, much work is still required to elucidate whether, and under what circumstances, C starvation may actually occur in nature. Note that we do not argue against the CSH as a mechanism leading to drought-induced mortality. Additional careful research designed to separate the effects of hydraulic failure from C starvation could reveal drought-induced C starvation before mortality. We predict this would be more likely in small seedlings or in plants with relatively limited total stored C pools. Our argument is against invoking this hypothesis as the most parsimonious causal explanation for drought-induced tree mortality in the absence of evidence against hydraulic failure and/or biotic interactions. Because the CSH is appealing and logical, implied causation could lead to its premature acceptance without the necessary tests to rule out other potential mechanisms, which could have negative consequences on the advancement of the field.

Carbon starvation in woody plants is not unprecedented. For instance, low atmospheric CO_2 concentration (between 180 and 220 ppm) during the last glacial period apparently caused C starvation in *Juniperus* in southern California (Ward *et al.*, 2005). However, modelled relative humidity during the same period was *c.* 10% higher than today, suggesting that C starvation occurred in response to low atmospheric CO_2 and not to drought. In fact, as we argue in the following section, water stress could be the limiting factor that prevents drought-induced C-reserve exhaustion.

Assumptions and contingent hypothesis

Drought-induced C starvation resulting from reserve exhaustion as stated by the CSH requires continued mobilization and translocation of stored C under drought. Therefore, an implicit, unstated assumption of the CSH hypothesis is that these processes are not affected by water stress. While substantial advancements have recently been made on our understanding of phloem physiology, fundamental aspects remain to be resolved (van Bel & Hafke, 2005; Thompson, 2006). For instance, Münch (1930) proposed that long-distance phloem transport occurs by bulk flow driven by hydrostatic pressure gradients between sources (high pressure) and sinks (low pressure). While this model remains largely intact and widely accepted, we still do not know the magnitude of the pressure differentials necessary for long-distance transport, the distances over which such pressure differentials are regulated, and the precise mechanisms involved (Thompson, 2006). That is, we still cannot describe the mechanistic basis by which long-distance phloem transport occurs in plants (an equivalent situation would be a lack of a mechanistic understanding of blood circulation in chordates!). Such a remarkable void results from our limited knowledge of phloem anatomy and from the fact that it is exceedingly difficult to measure phloem flow and pressure gradients nonintrusively. Given such a lack of foundation, it is not surprising that the relationship between phloem physiology and environmental stress has been largely ignored. Based on what is known to date, however, we argue that the assumption that C mobilization and phloem transport are not limited under water stress does not hold true (Fig. 1).

For instance, although drought reduces C assimilation, reduced rates of C export from leaves under drought (Sung & Krieg, 1979; Deng *et al.*, 1990; Quick *et al.*, 1992) have been attributed to negative effects of drought on phloem function (Deng *et al.*, 1990). Consistent with this, Ruehr *et al.* (2009) found that drought in *Fagus sylvatica* reduced the coupling between canopy photosynthesis and C transport to below ground, an effect associated with decreased phloem transport velocity and reduced rates of carbohydrate export from leaves. By a process of elimination, the authors suggested that drought effects on phloem transport were responsible for the decreased C transport to below ground. In trees, drought has also been shown to decrease phloem sap turgor in *Eucalyptus globulus* (Cernusak *et al.*, 2003). Consistently, in *E. globulus* phloem bleeding was noted to decrease with decreasing xylem water potential (Cernusak *et al.*, 2003) and occasionally to cease completely under severe water stress (Pate *et al.*, 1998). Under water stress, the maintenance of sufficient phloem turgor pressure to drive phloem transport is accomplished via osmotic adjustment and often associated with increased activity and/or expression of phloem-specific transport compounds (Aloni *et al.*, 1986; Pommerrenig *et al.*, 2007). Consistently, several studies have reported an increase in phloem sap sugar concentration in trees as water becomes limiting (Pate & Arthur, 1998; Gessler *et al.*, 2001; Cernusak *et al.*, 2003). Further, the C isotope ratio of the phloem sugar is often strongly and positively correlated with the C isotope ratio of recently assimilated sugars (Pate & Arthur, 1998; Pate *et al.*, 1998; Cernusak *et al.*, 2003; Keitel *et al.*, 2006), suggesting that newly assimilated C is used for phloem turgor maintenance. Interestingly, Pate & Arthur (1998) noted that trees with the highest recorded phloem sugar concentrations and less negative C isotope ratios during the dry season subsequently died. This suggests that under severe water stress, reduced C assimilation combined with low apoplastic water potential may impose a limit to phloem turgor maintenance beyond which phloem transport is impaired. In summary, studies to date indicate that recently assimilated sugars are involved in phloem osmotic adjustment as water becomes limiting, but that turgor maintenance is possible only up to a limit beyond which additional water stress causes a decrease in phloem turgor and transport rates.

Mobile carbohydrate compartmentalization away from sites of phloem loading has also been suggested as a possible

mechanism responsible, at least in part, for reductions of sugar export from leaves under drought (Quick *et al.*, 1992). This raises the possibility that, under water stress, stored C pools become unavailable for transport. There is some evidence that most plants fail to use all carbohydrate reserves following herbivory (Chapin *et al.*, 1990 and references therein) or deep shade, even when it leads to mortality (Piper *et al.*, 2009). Similarly, Millard *et al.* (2007) noted that nonstructural carbohydrate (NSC) pools in trees are never fully depleted to the extent that nitrogen (N) storage pools are, and that even girdling the phloem does not deplete NSC in roots completely. Possible mechanisms proposed to explain the failure of plants to use stored C pools include reduced accessibility to stored C pools over time as a result of their compartmentalization in tissues where they cannot be retrieved (Chapin *et al.*, 1990; Millard *et al.*, 2007), and constraints on enzymatic activity, such as the limited enzymatic access to the inner nucleus of starch granules (Srichuwong & Jane, 2007). In this case, storage pools become sequestered or inaccessible and, by definition, are no longer storage pools. This adds yet other layers of complexity to test the CSH. First, what fraction of mobile C compounds in plants is readily accessible at any given time and therefore constitutes an accessible reserve pool? Second, is this fraction constant over time or does water stress limit access to storage pools, thus rendering them inaccessible? The latter possibility is consistent with the results of Carpenter *et al.* (2008) where regrowth of *Salix nigra* after clipping treatments was reduced in plants subjected to water stress relative to controls, an effect that was related to a lower consumption of stored carbohydrates under water stress.

Another issue that deserves careful consideration is the potential of plants to down-regulate C demands under water stress via shedding of high-maintenance-cost tissues combined with down-regulation of respiration rates (acclimation responses). Mortality of fine roots and leaf shedding in response to drought (Rojas-Jimenez *et al.*, 2007; Chaves *et al.*, 2009) are expected to reduce C demand. While loss of leaf area reduces the potential for C assimilation, leaf shedding in response to drought may improve overall plant C balance, if C assimilation in water-stressed leaves is not sufficient to offset costs of respiration (i.e. leaves are shed when their own costs do not offset the overall benefit to the whole plant). Such a response is consistent with recent studies showing that leaf life span is tightly regulated to optimize overall plant C balance (Oikawa *et al.*, 2008; Reich *et al.*, 2009). Drought-induced decreases of leaf area could also ameliorate whole-plant water relations and C assimilation by increasing the proportion of water transport tissues (roots and sapwood) relative to leaves (Chaves *et al.*, 2009). In addition to tissue shedding, down-regulation of respiration rates has been shown to occur in response to increased temperature (Bryla *et al.*, 2001; King *et al.*, 2006) and it is the most common (although not unique) response to drought (reviewed by Atkin & Macherel, 2009). Although such reductions are small relative to drought-induced decreases of photosynthesis, their combined effect with that of tissue shedding could significantly contribute to overall reduced C demand under water stress. If so, adjustments of C demand under drought could reduce the likelihood of C starvation, particularly if residual stored C pools are sufficient to meet adjusted demands.

Many possible mechanisms, all driven directly or indirectly by climate change, can potentially explain the recent worldwide increase in tree dieback, either as single factors or in combination. Here, however, we focus on potential direct effects of drought on tree physiology that have been ignored so far. A significant body of literature suggests that mature trees under ambient CO_2 concentrations have considerable pools of stored C reserves which can meet substantial C demands even in the absence of C assimilation (Hoch *et al.*, 2003; Körner, 2003; Würth *et al.*, 2005; Millard *et al.*, 2007). Surprisingly, this body of knowledge has been largely ignored in the literature invoking drought-induced mortality as a result of C starvation. Yet we believe it is of fundamental relevance to this discussion. If mature trees have large pools of stored C, and if such pools increase with tree size and in drier sites, as suggested by Sala & Hoch (2009), then whether and under what circumstances these pools could become depleted to the point of inducing C starvation cannot be ignored. Estimates of how long stored C pools could sustain growth in the absence of photosynthesis vary widely (Ludovici *et al.*, 2002; Hoch *et al.*, 2003; Würth *et al.*, 2005). However, the fact that these pools may not be fully depleted, even after severe water stress leading to tree mortality (Bréda *et al.*, 2006), suggests that water stress limits the conversion of stored C pools into mobile forms of C, their respective mobilization to sites of phloem loading, or their long-distance transport. If mobilization of stored C and subsequent phloem transport is limited under water stress, cellular-level C starvation may occur irrespective of available stored reserves (Fig. 1). In this case, the actual direct physiological mechanism underlying drought-induced tree mortality (in the absence of hydraulic failure or biotic agents) is not C-reserve exhaustion as proposed by the CSH but rather water stress-impaired C metabolism and translocation. We believe that, from a physiological perspective, this distinction is critical because it involves fundamentally different mechanisms at the whole-plant level (Fig. 1) with important implications. For instance, the CSH as stated by McDowell *et al.* (2008) predicts that time to mortality under drought is a function of the size of a tree's C-reserve pool, which is clearly not the case if drought impairs C metabolism and transport. Therefore, premature acceptance of the CSH (Adams *et al.*, 2009) detracts from much-needed research in critical areas of tree physiology to fully understand tree, forest and ecosystem responses to global change.

Conclusions

The recent worldwide increase in tree dieback is most likely driven directly and indirectly by global climate change and associated drought. The hydraulic framework proposed by McDowell *et al.* (2008) to explain direct climate effects on tree physiology and mortality is very appealing, but it relies on several untested assumptions. In particular, we caution against acceptance of the CSH as a general mechanism to explain drought-induced mortality in the absence of hydraulic failure and biotic interactions without the necessary direct evidence. Our argument is based on the lack of direct evidence and on the fact that current research strongly suggests that the fundamental implicit assumption that C metabolism and/or long-distance transport is not affected by water stress does not hold true. The latter is supported by several factors, including evidence of large stored C pools in trees regardless of climate and past stress; lack of evidence that water stress consistently results in C depletion and C limitation; lack of evidence for C-reserve exhaustion preceding tree mortality under severe water stress; and the fact that evidence of C starvation is limited only to situations where water relations are not necessarily impaired. Clearly, trees whose tissues are not supplied with enough C to support their activity will starve and die. However, whether such C starvation occurs because of reserve exhaustion or because of impairment of long-distance transport is not an issue of semantics but a critical issue in plant physiology, with fundamental implications for plant ecology and evolution. Phloem transport lies at the core of plant resource allocation and life history, which in turn have consequences on community interactions and the evolution of plant traits under different environmental conditions. However, the knowledge void in phloem physiology precludes us from fully appreciating its critical role. Impaired phloem physiology could very well be involved in the recent worldwide drought-induced mortality. Premature acceptance of the CSH as formalized by McDowell *et al.* (2008) as the principal mechanism could divert research away from critical areas needed to fully understand forest ecosystem responses to global change.

Acknowledgements

The Chilean government (Department of International Relationships of the Comisión Nacional de Ciencia y Tecnología, CONICYT) provided funds for an international collaboration among the authors which led to this paper. We thank three anonymous reviewers and J. Martínez-Vilalta for their constructive feedback.

Anna Sala[1]*, Frida Piper[2] and Günter Hoch[3]

[1]Division of Biological Sciences, The University of Montana, Missoula MT-59812, USA;
[2]Centro de Investigación en Ecosistemas de la Patagonia, Bilbao 449, Coyhaique, Chile;
[3]Institute of Botany, University of Basel, Schönbeinstrasse 6, CH-4056 Basel, Switzerland
(*Author for correspondence: tel +406 243 6009; email sala@mso.umt.edu)

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Key words: carbon pools, carbon starvation, drought, hydraulic constraints, phloem transport, reserves, tree mortality.

The UNITE database for molecular identification of fungi – recent updates and future perspectives

Rationale

Ectomycorrhizal (ECM) fungi are typically examined for taxonomic affiliation through sequence similarity searches involving the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit and the International Nucleotide Sequence Databases (INSD) (Peay *et al.*, 2008; Taylor, 2008; Benson *et al.*, 2009; Tedersoo *et al.*, 2010). However, the usefulness of these searches is constrained by the technical quality and the taxonomic reliability of the reference sequences in the databases (Nilsson *et al.*, 2006; Bidartondo, 2008). The meagre data on voucher specimen, country of collection and host, which are associated with many of the entries, place a further restriction on the usefulness of the entries in an ecological or taxonomic context (Ryberg *et al.*, 2009). The UNITE project (Kõljalg *et al.*, 2005) was initiated in 2001 to address these problems through a free online database for high-quality reference records of ITS sequences from North European ECM fungi. Taxonomic reliability was the founding principle of the initiative; all records were determined to species level (or as far as possible) by researchers well versed in the taxonomic group in question, and all sequences were obtained from, or in association with, richly annotated fruiting bodies (voucher specimens) deposited in public herbaria.

The years since the 2005 publication of UNITE have witnessed a proliferation of environmental sequencing efforts from all over the world, and there is a clear tendency in the recent literature to target entire communities of fungi rather than individual taxa or subsets of the full diversity. Such studies may still focus on ECM fungi, but inherent to many of these projects is a desire to examine also the non-ECM sequences to obtain a better view of the trophic processes and potential interdependence or interactions among taxa (Lindahl *et al.*, 2007). Any modern initiative aiming to provide facilities for sequence identification must therefore be prepared for geographically diverse, and ecologically disparate, query sequences. These observations, together with the prospects of emerging high-throughout sequencing technologies such as massively parallel (454) pyrosequencing (Margulies *et al.*, 2005; Shendure & Ji, 2008; Hibbett *et al.*, 2009), suggest that a sequence database with a limited geographical or nutritional coverage of taxa – and where sequences are processed one at a time – may no longer serve the needs of the research community in a fully efficient way. We have been working to keep the UNITE database abreast of developments in the field, and in the present Letter we list the major updates in technology, methodology and policy that UNITE has undergone since its initial publication. A set of guidelines for its future development is also provided.

Sequence coverage and taxonomic inclusiveness: statistics and policy updates

The number of voucher-associated sequences in UNITE has increased from 811 in 2005 to nearly 3000 at present (Table 1). The number of species of North European ECM fungi represented in UNITE has more than doubled to 896 (73% of the known ECM fungi in North Europe; Hansen & Knudsen, 1997; Knudsen & Vesterholt, 2008), and the total number of species has increased from 480 to 1078. Taxon sampling is necessarily not uniform, but reflects the availability of taxonomic expertise and updated generic revisions. For example, *Ramaria* (two species in UNITE), *Pezizales* (30 species in UNITE) and *Helotiales* (32 species in UNITE) are taxa for which a substantial amount of data remain to be generated; by contrast, sequences for 80% of the known North European species of *Lactarius* and *Boletus* have been deposited in UNITE.

The first major change to announce is that the previous geographical and ecological restrictions on the scope and sequence coverage of UNITE have been lifted. Although we will continue to expand and enhance the taxon sampling of mycorrhizal fungi, we now accept fully identified and well-annotated reference sequences from any geographical locality, nutritional mode and group of fungi, as long as they are supported by vouchers or type cultures, and the sequence authors have a documented expertise of the taxa in question. The curators of UNITE reserve the right to send new reference sequences for peer review. Unidentified and environmental sequences may also be deposited in UNITE, provided that they are of high quality, well annotated and mutually nonredundant (i.e. large sets of identical sequences from the same author and study site will not be accepted); in addition they must cover the ITS region in full. The option to submit reference sequences with the sequence data open to query, but with the species name withheld (i.e. 'locked'), will be allowed

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Table 1 Statistics on the records of the UNITE database as of November 2009

	Taxon name	No. of sequences 2009	No. of species 2009	No. of sequences March 2005	No. of species March 2005
Basidimycota	Agaricales	1576	587	330	227
	Boletales	291	102	133	62
	Cantharellales	18	8	10	7
	Dacrymycetales	1	1	0	0
	Geastrales	2	2	0	0
	Hymenochaetales	1	1	0	0
	Hysterangiales	2	2	0	0
	Phallales	8	7	2	2
	Polyporales	56	27	7	5
	Russulales	442	191	216	115
	Thelephorales	275	78	98	49
	Tremellales	11	5	3	2
Ascomycota	Eurotiales	4	2	3	2
	Helotiales	37	32	0	0
	Hypocreales	1	1	0	0
	Lecanorales	1	1	0	0
	Orbiliales	1	1	0	0
	Pezizales	33	30	9	9
Total		2760	1078	811	480

Where applicable the corresponding statistics from 2005 are also given. A list of all species and the number of sequences present in UNITE is available at http://unite.ut.ee/SearchPages.php

only when the sequences have been accepted for publication but are not yet online. We are furthermore looking into supporting the provision of operational names of the accession number type for clusters of hypothetically conspecific sequences that cannot be identified to species level. Such informal names represent an unambiguous way of referring to such taxa until the data are there to warrant formal description of the species.

Although UNITE will maintain a focus on the ITS region, there is now generic support for any other gene and genetic marker pertinent to the identification of fungi. The nuclear large subunit (nLSU/28S) gene, arguably one of the mainstays of current fungal phylogenetic inference, is an example of this. Although not always discriminative at the species level, the nLSU can be used, in the absence of a good ITS match, to assign specimens to a higher taxonomic level. The number of nLSU sequences in UNITE is modest, but several sizable data sets of primarily saprophytic fungi have been scheduled for inclusion in the near future. We hope that these will be followed by more, and invite the mycological community to deposit nLSU sequences in UNITE. It is important that such data be accompanied by primer and amplification details, if this information is not available in a tagged publication.

Richer and more dynamic project-oriented relational database model

The initial database model has been expanded into a 130-table SQL-compliant database structure compatible with the Taxonomic Database Working Group standards (http://www.tdwg.org/standards/). The structure draws from Taxonomer (Pyle, 2004; and subsequent additions) to capture the full complexity of modern mycological taxonomy and nomenclature. Metadata pertaining to sequences or sets of sequences can now be stored in a way open to direct query and include locality, habitat, soil type and host (Supporting Information Fig. S1). Sequence sets can be formed to reflect contexts such as studies, plots and samples, and the sequences in such sets can be addressed jointly, separately, or in combination with all other sequences. Particular care has been taken to make sure that information can be represented in a fully nuanced way through many-to-many relations: a sequence can have more than one correct name (to account for anamorph–teleomorph relationships and synonyms), a species can have many habitats and ecological characteristics, and a study may be composed of any number of distinct or coupled plots and subplots. A researcher may, for instance, divide the sequences from some given project into sets – reflecting, for example, host or plot of origin – and compare these for differences in taxonomic composition and species richness. Research groups can be granted far-reaching access to the system, allowing, for example, tailoring of the submission procedure in the interest of exact and efficient storage and representation of particularly complex data. Indeed, we envision UNITE as being a fully fledged sequence-management system that individual researchers or research groups can use to store and analyze data from entire projects or study sites. Many of the features of such

a system are already in operation. We feel that the inclusion of this sequence-management environment to process new and existing sequences distinguishes the UNITE database from the INSD.

Improved support for storage of auxiliary data

It is now possible to associate any number of binary files with sequences, species, studies, or other objects or contexts within UNITE. While this service was initially conceived as a response to the debate on the availability of primary sequence data such as chromatograms and other raw sequence data (Costello, 2009), any noncopyrighted and freely available file relevant to the interpretation of the underlying or downstream data will be accepted. This includes, but is not limited to, photographs of fruiting bodies or root tips, drawings of spores and mantle structure, maps, GIS (geographic information system) data files and PDF (Portable Document Format) documents; size restrictions may, however, apply and the data authors are requested to use file formats with manageable file sizes. Scientific publications may be deposited for public view alongside sequence data as long as no copyright laws or legal limitations in distribution or dissemination are violated: it is the responsibility of the depositor/author to obtain such permission where needed. We are also willing to hyperlink to relevant external files provided that these are maintained by – or otherwise under the control of – the sequence author in question and that a reasonable permanency can be guaranteed (Ducut *et al.*, 2008). All such links are checked for validity every 6 months.

New sequence submission and maintenance procedure, as well as improved INSD connectivity

The sequence-deposition process in UNITE has been reworked and now features a log-in system through which the user can deposit and annotate even large sets of sequences. Information can be updated by the user (sequence author) through the log-in system; any such change will take effect immediately. Users are not allowed to modify the records of other sequence authors, but a Wikipedia-style system for commenting on individual sequences is under development. There is a batch-submission system for environmental sequences, and a software package to examine fungal ITS sequences for the presence of chimeric elements is in the final stages of development. All sequence authors are encouraged also to submit their sequences to the INSD; UNITE is now an INSD LinkOut provider, such that all sequences in INSD that are also present in UNITE (possibly with richer annotation) are hyperlinked there. UNITE similarly offers the possibility to link entries to the INSD. UNITE exchanges data with the INSD on a trimestrial basis and keeps a local copy of all fungal ITS sequences in INSD (approximately 135 000 sequences belonging to some 13 500 fully identified species as of July 2009). The fully identified sequences from INSD can be included in, or excluded from, sequence queries in UNITE. Similarly, environmental/unidentified sequences from UNITE and INSD can be excluded from searches.

Usage statistics as a window on the road ahead

UNITE (Kõljalg *et al.*, 2005) has been cited about a hundred times since publication, with 2008 being the year with the highest number of citations (30 in total). The studies citing UNITE cover all five continents. The proportion of users from fields other than mycorrhizal and systematic mycology is growing. This increases the pressure on UNITE to provide information that is clear, accurate and up-to-date; the information should ideally be presented with a general scientific, rather than a strictly mycological, target audience in mind. It is equally clear that in future many users will not turn to UNITE with one or a handful of sequences for query and analysis, but with hundreds or thousands. This trend is taken to its extreme by the 454 pyrosequencing platform, whose voluminous output forms a challenge to any database effort (Buée *et al.*, 2009; Jumpponen & Jones, 2009; Öpik *et al.*, 2009). We do not currently envision UNITE as a full solution for newly generated, unprocessed raw sequence data from 454-based projects, but we will seek to make it a swift and useful resource for analysing pre-processed, clustered 454 data sets. As a first step we are preparing a new batch BLAST search function for joint analysis of multiple query sequences. A second, and more challenging, step is to employ phylogenetic analysis in the batch-mode identification process, but the details remain to be formalized here.

The pursuit of mycological knowledge is a global scientific enterprise. UNITE collaborates with the Fungal Environmental Sampling and Informatics Network (FESIN) (Bruns *et al.*, 2008; Horton *et al.*, 2009) to establish guidelines and standards for how environmental samples of fungi should be processed and analysed. Much will be gained in terms of time and resources if software and infrastructural development can be co-ordinated. Furthermore, the geographical coverage of UNITE and FESIN together is considerable and should lead to a significant leap in the number of reference sequences in UNITE over the next few years. The challenges remain substantial, however, and we welcome assistance and collaboration to further the underlying objectives and help to bridge the gap between mycology and other disciplines. We invite any researcher or research group with data or resources relevant to reliable molecular identification of fungi to either deposit their data in UNITE or to contact the UNITE team for further discussions. We similarly invite anyone with a set of fungal

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sequences in need of taxonomic assignment to consider the sequence-processing environment of UNITE and to make any information that would cast further light on data already residing in the database available to the scientific community. We have secured at least basic funding for UNITE for the foreseeable future, and we intend the database to be a permanent resource for the scientific community.

Kessy Abarenkov[1], R. Henrik Nilsson[1,2], Karl-Henrik Larsson[3], Ian J. Alexander[4], Ursula Eberhardt[5], Susanne Erland[6], Klaus Høiland[7], Rasmus Kjøller[8], Ellen Larsson[2], Taina Pennanen[9], Robin Sen[10], Andy F. S. Taylor[11], Leho Tedersoo[1], Björn M. Ursing[12], Trude Vrålstad[7], Kare Liimatainen[13], Ursula Peintner[14] and Urmas Kõljalg[1]*

[1]Department of Botany, Institute of Ecology and Earth Sciences, University of Tartu, 40 Lai St, 51005 Tartu, Estonia; [2]Department of Plant and Environmental Sciences, University of Gothenburg, Box 461, 405 30 Göteborg, Sweden; [3]The Mycological Herbarium, Natural History Museum, University of Oslo, PO Box 1172, Blindern, N-0318 Oslo, Norway; [4]School of Biological Sciences, University of Aberdeen, Cruickshank Building, Aberdeen AB24 3UU, UK; [5]Centraalbureau voor Schimmelcultures, PO Box 85167, 3508 AD Utrecht, the Netherlands; [6]Max Planck Institute for Chemical Ecology, Hans-Knoell-Strasse 8, D-07745 Jena, Germany; [7]Department of Biology, University of Oslo, Box 1066, Blindern, N-0316 Oslo, Norway; [8]Biological Institute, Terrestrial Ecology, University of Copenhagen, Øster Farimagsgade 2D, DK-1353 Copenhagen, Denmark; [9]The Finnish Forest Research Institute, PL 18, FI-01301 Vantaa, Finland; [10]Department of Environmental and Geographical Sciences, Manchester Metropolitan University, John Dalton Building, Chester Street, Manchester M1 5GD, UK; [11]Macaulay Institute, Craigiebuckler, Aberdeen AB15 8QH, UK; [12]Beronem AB, Kullagränd 6, 239 42 Falsterbo, Sweden; [13]Department of Biology, University of Washington Seattle, Box 351330, 235 Johnson Hall, WA 98195, USA; [14]Institute of Microbiology, University Innsbruck, Technikerstr. 25, 6020 Innsbruck, Austria
(*Author for correspondence: tel +372 738 3027; email urmas.koljalg@ut.ee)

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Screenshot from the web-based workbench demonstrating how and what kind of metadata can be uploaded.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

Key words: environmental sequencing, fungi, molecular identification, sequence database, UNITE.

Polyphosphate has a central role in the rapid and massive accumulation of phosphorus in extraradical mycelium of an arbuscular mycorrhizal fungus

Introduction

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with most land plants and promote growth of the host through enhanced uptake of phosphate (Pi) (Smith & Read, 2008). It has been well documented that the high-affinity Pi transporters on the plasma membrane of extraradical hyphae play a main role in Pi uptake from soil (Harrison & Van Buuren, 1995; Maldonado-Mendoza *et al.*, 2001). On the other hand, the mycorrhiza-specific plant Pi transporters localized on the periarbuscular membrane are responsible for the uptake of Pi released from arbuscules (Rausch *et al.*, 2001; Maeda *et al.*, 2006; Javot *et al.*, 2007). Despite increasing knowledge of the membrane transport systems in the symbiotic associations, information about the mechanism of Pi translocation through AM fungal hyphae is quite limited. Evidence that AM fungi accumulate polyphosphate (polyP) in hyphae was first obtained more than three decades ago (Callow *et al.*, 1978). Polyphosphate is a linear chain of three to thousands of Pi residues linked by high-energy phosphoanhydride bonds and has been found in nearly all classes of organisms (Kornberg *et al.*, 1999). The compound has many functions in the cell, including acting as a Pi reservoir, an alternative energy source of ATP and a metal chelator (Kornberg *et al.*, 1999). Although polyP is suggested to be involved in long-distance Pi translocation through hyphae in AM associations (Cox *et al.*, 1980; Ezawa *et al.*, 2002), the physiological roles and behavior of the compound in the fungi are largely unknown. It has been reported that the compound consists of only a small part of total cellular phosphorus (P) in AM fungi: the proportions of polyP to total P were estimated as 16% in *Glomus mosseae* (Capaccio & Callow, 1982) and 5–17% in *Gigaspora margarita* (Solaiman *et al.*, 1999). These estimates suggest that Pi and/or other P compounds may play a more significant role in P storage/translocation in the fungi. On the other hand, Pi taken up by hyphae was converted to polyP quite rapidly (Ezawa *et al.*, 2004; Viereck *et al.*, 2004), and the rate of polyP accumulation was comparable to that of a polyP-hyperaccumulating bacterium found from activated sludge (Ezawa *et al.*, 2004). These observations led us to hypothesize that the fungi could potentially accumulate much larger amounts of polyP than previously reported. In particular, Viereck *et al.* (2004) provided a comprehensive view of the relative dynamics of various (soluble) P compounds in an AM fungal mycelium using the *in vivo* ^{31}P-NMR (nuclear magnetic resonance) technique, and suggested that polyP might be the largest P storage in the fungi. However, NMR-invisible P compounds, such as long-chain polyP and structural P, might be present in the cell, and the absolute (potential) pool size of the cell for polyP has not been estimated so far. Therefore, further quantitative study on the dynamic of polyP in AM fungi with respect to total cellular P is required. In addition, it was predicted that the maximum pool size for polyP would be demonstrable in P-starved AM fungi, which could accumulate polyP as rapidly as a polyP-hyperaccumulator (Ezawa *et al.*, 2004). In the present study, the dynamics of polyP, total P and Pi were investigated in an AM fungus grown under P-starvation conditions to clarify the significance of polyP in P storage/translocation in AM fungal associations.

Materials and Methods

Culture conditions

Lotus japonicus L. cv Miyakojima MG-20 (National Bioresource Project Legume Base, http://www.legumebase.agr.miyazaki-u.ac.jp/index.jsp) were sown on moistened filter paper in a Petri dish and germinated at 25°C for 2 d in the dark. Three seedlings were transplanted to the mycorrhizal compartment (MC) of a dual mesh bag culture system in a 230 ml plastic pot (7.6 cm diameter) and inoculated with *Glomus* sp. HR1 (MAFF 520076) at 500 spores per pot. The dual mesh bag system consisted of two main compartments – a MC and a hyphal compartment (HC) – that were separated by a cone-shaped dual nylon mesh bag (37 μm pore size, Nippon Rikagaku Kikai, Tokyo, Japan) (Supporting Information, Fig. S1). The MC was defined as the region inside the inner mesh bag (31 ml in volume, 5.2 × 4.5 (W × H) cm), and the HC (159 ml

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in volume) was defined as the region outside of the outer mesh bag (6.8 × 5.9 (W × H) cm). The medium in these compartments was autoclaved river sand. In between the inner and outer mesh bags, autoclaved subsoil with a high-P absorption coefficient (2.8 g P_2O_5 kg^{-1} soil, pH 5.0) was layered as a P-diffusion barrier (10 mm in width). The pore size of the nylon mesh was small enough to prevent *L. japonicus* roots from passing through, but large enough to allow passage of AM fungal hyphae. The seedlings were grown in a growth chamber equipped with fluorescent light at a photon flux density of 150 μmol m^{-2} s^{-1} (16 h photoperiod, 25°C) and thinned to two plants per pot at 1 wk after sowing. The plants (whole pot) received deionized water (DIW) every other day for the first week, then low-P nutrient solution (4 mM NH_4NO_3, 1 mM K_2SO_4, 75 μM $MgSO_4$, 2 mM $CaCl_2$, 50 μM Fe-EDTA and 50 μM KH_2PO_4) from the second to the sixth week, and nonP nutrient solution (KH_2PO_4 was withheld from the low-P nutrient solution) for the seventh week in sufficient amount until the solution flowed out from the drain. At the beginning of eighth week, a 1 mM Pi (KH_2PO_4) solution was applied using a pipette to the HC gently in a sufficient amount until the solution flowed out from the drain, and then the Pi solution was washed out by applying DIW with a watering can in a sufficient amount 1 h after Pi application. Mycorrhizal roots and extraradical mycelium in the MC and mycelium in the HC were harvested separately after Pi application at 1 h intervals as follows. The MC (inner mesh bag) was taken off the pot and transferred to water, and then mycorrhizal roots and attaching mycelium (roots + mycelium) were collected after removing adhering sand particles by gentle shaking in the water. Detached mycelium in the water was further collected by the wet sieving and combined with the roots + mycelium fraction. Extraradical mycelium in the HC was collected by the wet sieving after removing the P-diffusion barrier (outer mesh bag). The samples were blotted with a paper towel, frozen in liquid nitrogen immediately and stored at −80°C.

Analytical procedures

In the case of mycelium from the HC, 5–30 mg (FW) of material was ground in an ice-cooled mortar and pestle with 10- to 20-fold volume (w/v) of extraction buffer (8 M urea : 50 mM Tris-HCl, pH 8.0) and transferred to a 1.5 ml tube. In the case of roots + mycelium from the MC, 0.7–2.0 g (FW) of material was ground in a mortar with liquid nitrogen and mixed with fivefold volume (w/v) of the extraction buffer, and then 500 μl of the slurry was transferred to a 1.5 ml tube.

For determination of total P in the HC samples, 100 μl of the slurry was transferred to a 14.7 ml Teflon vial (Savillex, Minneapolis, MN, USA), mixed with 2.5 ml of 1.8 M sulfuric acid, heated at 250°C for 2 h to evaporate water (until sulfuric acid was concentrated) and then digested at 250°C for 1 h by using 0.2–0.3 ml hydrogen peroxide as an oxidant. Pi concentrations in the digests were determined by the ascorbic acid method (Watanabe & Olsen, 1965). PolyP concentrations in the slurries prepared from the HC and MC samples were determined by the reverse reaction of polyphosphate kinase (PPK), as described by Ezawa *et al.* (2004). Free Pi concentrations in the slurries from the HC samples were determined by the ammonium molybdate method (Ohnishi *et al.*, 1975) using 200 μl supernatant obtained after centrifuging the slurry at 14 000 ***g*** for 15 min. Ten microliters of the slurry from the HC and MC samples was taken for the determination of protein concentration using the DC Protein Assay Kit (Bio-Rad Laboratories, Tokyo, Japan) with bovine serum albumin as standard.

Experimental setup and data analysis

The time course analysis (from 0 to 9 h after Pi application) of total P and that of polyP in the HC were conducted separately using different batches of plant/fungal material. For these analyses, 22 pots were prepared as one batch, and mycelial samples collected from two pots grown in the same batch were combined as one sample (5–30 mg FW per sample). One set of time course experiments (from 0 to 9 h after Pi application) was conducted using one batch (without replication) and triplicated using three independent batches. For data analysis, average values were calculated from the data obtained from the three replicated experiments (n = 3). For the time course analysis of polyP in roots + mycelium in the MC (from 0 to 10 h after Pi application), 33 pots were prepared as one batch, and three samples (0.7–2.0 g FW per sample) harvested from three pots were analyzed separately (n = 3). For the simultaneous analysis of total P, polyP and Pi in the HC (from 0 to 6 h after Pi application), 22 pots were prepared as one batch, and mycelial samples collected from two pots grown in the same batch were combined as one sample. One set of time course experiments (from 0 to 6 h after Pi application) was conducted using one batch (without replication) and replicated five times using five independent batches. For data analysis, average values were calculated from the data obtained from the five replicated experiments (n = 5).

Analysis of variance (ANOVA) with the Tukey–Kramer test as a post-hoc test or Student's t-test was applied for data analysis using the StatView software (SAS Institute Inc., Cary, NC, USA).

Results

The total P content of extraradical mycelium in the HC was 3.6 μmol mg^{-1} protein at time zero, which increased to 8.2 μmol mg^{-1} protein 5 h after Pi application and then

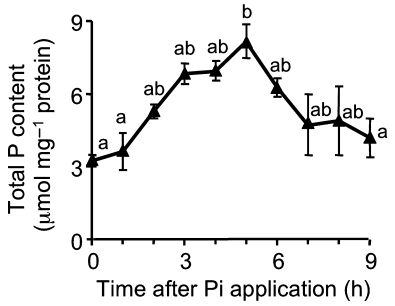

Fig. 1 Time course assessment of total phosphorus (p) content in the extraradical mycelium of *Glomus* sp. HR1 in the hyphal compartment. One millimolar phosphate (Pi) solution was applied to the hyphal compartment at time zero and washed with deionized water 1 h after Pi application. Average values (± SE) obtained from three independent experiments (n = 3) are shown. Different letters indicate significant differences (P < 0.05, Tukey–Kramer test).

decreased to 5.2 μmol mg^{-1} protein 9 h after Pi application (Fig. 1). The polyP content of extraradical mycelium in the HC increased from 0.5 to 7.1 μmol mg^{-1} protein from 0 to 6 h after Pi application and decreased to 2.8 μmol mg^{-1} protein by 9 h after Pi application (Fig. 2). The apparent accumulation rates of total P and polyP from 0 to 5 h after Pi application were 1.03 and 1.14 μmol mg^{-1} protein h^{-1}, respectively, and were not significantly different (t-test, P < 0.05). The apparent declining rates of total P and polyP from 5 to 9 h after Pi application were 0.94 and 1.06 μmol mg^{-1} protein h^{-1}, respectively, and were also not different at P < 0.05. The polyP content of mycorrhizal roots + extraradical mycelium in the MC was maintained within a range of 3.8 to 6.6 nmol mg^{-1} protein from 0 to 5 h after Pi application, increased to 44.3 nmol mg^{-1} protein from 6 to 9 h after Pi application and then decreased to 9.8 nmol mg^{-1} protein 10 h after Pi application (Fig. 3). To investigate whether Pi and/or unknown precursors of polyP were accumulated before polyP accumulation, the content of free Pi, total P and polyP of extraradical mycelium in the HC was measured simultaneously. Total P and polyP content increased synchronously from 2.9 to 6.7 μmol mg^{-1} protein and from 0.2 to 4.3 μmol mg^{-1} protein, respectively, from 0 to 5 h after Pi application (Fig. 4). By contrast, free Pi content remained constant in the range 200–500 nmol mg^{-1} protein during the experiment. It is noteworthy that the concentration of polyP reached 64% of total P 5 h after Pi application. Differences between amounts of total P and polyP were constant from 0 to 6 h after Pi application (Fig. 4).

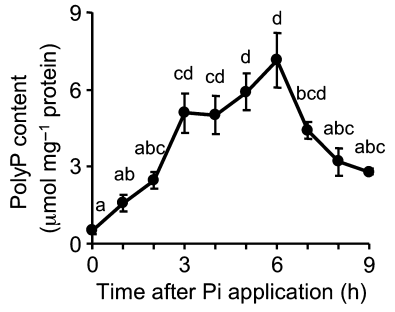

Fig. 2 Time course assessment of polyphosphate (PolyP) content in the extraradical mycelium of *Glomus* sp. HR1 in the hyphal compartment. One millimolar phosphate (Pi) solution was applied to the hyphal compartment at time zero and washed with deionized water 1 h after Pi application. Average values (± SE) obtained from three independent experiments (n = 3) are shown. Different letters indicate significant differences (P < 0.05, Tukey–Kramer test).

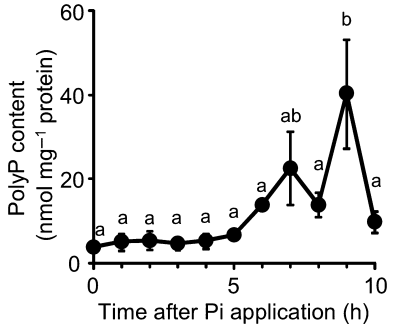

Fig. 3 Time course assessment of polyphosphate (PolyP) content in the *Lotus japonicas–Glomus* sp. HR1 mycorrhizal roots and extraradical mycelium in the mycorrhizal compartment. One millimolar phosphate (Pi) solution was applied to the hyphal compartment at time zero and washed with deionized water 1 h after Pi application. Average values (± SE; n = 3) obtained from one representative experiment are shown. Different letters indicate significant differences (P < 0.05, Tukey–Kramer test).

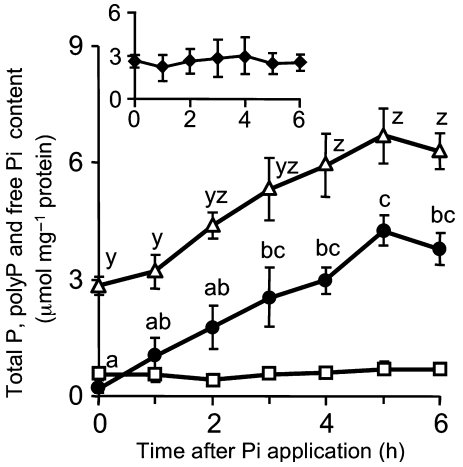

Fig. 4 Time course assessment of total phosphorus (P, triangles), polyphosphate (PolyP, circles) and free phosphate (Pi, squares) content in the extraradical mycelium of *Glomus* sp. HR1 in the hyphal compartment. One millimolar phosphate (Pi) solution was applied to the hyphal compartment at time zero and washed with deionized water 1 h after Pi application. The average values (± SE) obtained from five independent experiments (n = 5) are shown. Different letters indicate significant differences (P < 0.05, Tukey–Kramer test). Inset: graph shows differences between the amounts of total phosphorus and polyphosphate during the time course analysis (the units are the same as those in the large graph). No significant difference was observed between the amounts at all time points (P > 0.05, ANOVA).

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Discussion

The present study demonstrated that the AM fungus was capable of accumulating polyP > 60% of total cellular P, implying that the potential pool size of polyP in the cell was much larger than previously considered. Rapid and massive accumulation of polyP in microorganisms was first discovered in yeast *Saccharomyces cerevisiae* more than four decades ago and defined as 'polyP overplus (or overcompensation)' (reviewed in Harold, 1966); for example, the level of polyP in *S. cerevisiae* that was grown under P-deficient conditions increased 20-fold 2 h after resupply of Pi, up to 38 mg g^{-1} DW (corresponding to 400 μmol g^{-1} DW) (Trilisenko *et al.*, 2002). In the present study, *Glomus* sp. HR1 was found to respond to Pi similarly: the amount of polyP in the fungus grown under P-deficient conditions increased 14-fold 5 h after Pi application, up to 7.1 μmol mg^{-1} protein, corresponding to 390–500 μmol g^{-1} DW (the values were estimated based on the following parameters: protein content, 11–15 mg g^{-1} FW; water content, 80%). Although this was achieved by the application of quite a high concentration of Pi that rarely occurs under natural conditions, the amount is comparable to that observed in the yeast 'polyP overplus' and much higher than previously reported in AM fungi: *G. mosseae* intraradical hyphae, 32 μmol g^{-1} DW (Capaccio & Callow, 1982); *G. margarita* extraradical hyphae, 32–48 μmol g^{-1} DW (Solaiman *et al.*, 1999) (estimated on the assumption that water content was 80%). It has been considered that 'polyP overplus' would be the feature evolved in a wide range of microorganisms to prepare P deficiency, because Pi availability tends to be low in natural environments (Harold, 1966). Glomeromycotan fungi may have acquired the traits involved in 'polyP overplus' during the early evolution and could successfully provide a great competitive advantage in P acquisition for their host plants by facilitating a large P pool.

Our observations that total P and polyP increased and decreased synchronously without fluctuation in the amount of free Pi suggest that neither Pi nor an intermediary P metabolite such as short-chain polyP or organic P compound, which could be detected by the total P measurement but not by the PPK/luciferase method, was accumulated before polyP accumulation. This supports the idea that polyP formation in AM fungi is quite rapid (Ezawa *et al.*, 2004) and thus contributes to maintaining cellular Pi at a low concentration for efficient uptake of Pi (Viereck *et al.*, 2004), confirming that polyP plays a significant role in AM fungal P metabolism as a temporary but largest P storage.

Polyphosphate first increased in extraradical mycelium in the HC and later in mycorrhizal roots + mycelium in the MC. These observations were consistent with those reported by Viereck *et al.* (2004) and strongly suggest that polyP mediates long-distance P translocation through hyphae. In this context, our experimental system could provide further information about parameters for P translocation in AM symbiosis: the declining rates of polyP in mycelium in the HC from 5 to 9 h after Pi application (1.06 μmol mg^{-1} protein h^{-1}, corresponding to 58–80 μmol g^{-1} DW h^{-1}) can be regarded as the apparent P-translocation rate through hyphae towards the plants. In addition, the time lag of 5–6 h for the increases in polyP level in roots + mycelium in the MC can be regarded as the time required for P to pass the 10 mm diffusion barrier, that is, polyP (or Pi) moved towards the plant at 1.7–2.0 mm h^{-1}. In the *in vitro* carrot root-organ–*G. intraradices* association, it took 14 h to translocate radiolabeled P from extraradical to intraradical hyphae (Nielsen *et al.*, 2002), suggesting that the processes of P accumulation/translocation *in vitro* were slower than those in our open culture system. Differences in experimental systems may affect the energy status of the fungal partner through carbon supply from the plant partner, and this may be reflected in the differences in the rates of P accumulation/translocation (Olsson *et al.*, 2002; Bücking & Shachar-Hill, 2005). It has been well documented that there are large inter- and intraspecific variations in the efficiency of P uptake by AM fungi (Jakobsen *et al.*, 1992; Maldonado-Mendoza *et al.*, 2001; Nielsen *et al.*, 2002; Munkvold *et al.*, 2004). In fact, the apparent polyP accumulation rate of *Archaeospora leptoticha* (Ezawa *et al.*, 2004) is more than twice as rapid as that of *Glomus* sp. HR1. Accordingly, the parameters for polyP accumulation/translocation presented in our study could be applicable for the assessment of inter- and intraspecific variations in P delivery potential among AM fungi.

In the present experimental system, polyP formation (a net increase in polyP) was observed even after the Pi-washing (removal) process conducted 1 h after Pi application. The washing step shortened the duration of Pi uptake by hyphae and was thus essential to estimate the declining rate of polyP in mycelia. Two reasons can be proposed to explain the prolonged Pi uptake after the washing process: first, the amount of DIW used for the washing was insufficient, and thus Pi remained in the medium; and second, Pi was captured in hydrophilic (viscous) material such as extracellular polysaccharide gels around hyphae, which might be secreted by the fungus and removed by the wet sieving but not by watering. The former is unlikely, because an increase in DIW for the washing process did not shorten the duration of Pi uptake. The fact that adherence of organic material and fine sand particles to mycelia was observed frequently suggests that extracellular polysaccharide is present around hyphae, and thus the latter seems likely.

This study demonstrated the significance of polyP as the largest P storage and a mediator of long-distance P translocation in AM fungi. It remains uncertain, however, whether P is translocated as polyP without turning over, or whether it is translocated as Pi through dynamic regulation

of polyP synthesis/hydrolysis (Ezawa *et al.*, 2001). Further investigations are required to understand the whole picture of the P-delivery system in the symbiotic associations.

Acknowledgements

The PPK-over expressing *E. coli* was a kind gift from Prof. Arthur Kornberg (Stanford University, deceased). We thank Dr Derek B. Goto (Hokkaido University) for critical reading of the manuscript. This work was supported by the Grant-in-Aid for Scientific Research (19380040) from JSPS (to TE).

Nowaki Hijikata[1], Masatake Murase[1], Chiharu Tani[1], Ryo Ohtomo[2], Mitsuru Osaki[1] and Tatsuhiro Ezawa[1]*

[1]Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589 Japan;
[2]NARO-NILGS, Nasushiobara, Tochigi 329-2793 Japan
(*Author for correspondence: tel +81 11 857 9732;
email tatsu@res.agr.hokudai.ac.jp)

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Dual mesh bag culture system.

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Key words: arbuscular mycorrhizal fungi, phosphorus translocation, polyphosphate overplus, symbiosis.

New Phytologist

Meetings

Effectors, effectors *et toujours des* effectors

22nd New Phytologist Symposium: Effectors in plant–microbe interactions, INRA Versailles, France, September 2009

Despite their diversity, microbes seem to have evolved similar strategies to interact with plants. One such strategy involves the production of effectors that were first identified in bacteria as proteins injected into plant cells by a specific molecular syringe. Since then, effectors have been identified in a variety of eukaryotic microbes and are now defined as molecules, produced by bio-aggressors, pathogens and symbionts during infection, that act on plant cells (Hogenhout *et al.*, 2009). The study of effectors is a rapidly expanding research field that benefits from recent advances in microbial and plant genomics. The 22nd New Phytologist Symposium aimed to support this research field by bringing together, in Versailles, scientists from around the world who were working on different aspects of plant–microbe interactions over a wide range of pathosystems (viruses, bacteria, fungi, oomycetes, nematodes and insects). The Symposium was timely and popular, even though it took place just a few months after the XIV International Congress on Molecular Plant–Microbe Interactions in Quebec, which had a strong emphasis on effector biology (Walton *et al.*, 2009). Indeed, this specialized New Phytologist Symposium translated this mainstream research on microbial effectors into lively discussion sessions.

The conference was covered with a live twitter feed (#NPS09 tag, at http://tinyurl.com/y877xoy). Here, we present some of the highlights, with a special mention of three posters that were selected for awards.

'…fungal symbionts use proteins secreted in host plant tissues to interact with their hosts, extending the importance of effectors from pathogens to beneficial microbes.'

Bacterial effectors act on a wide range of plant cellular processes

Pioneering research on bacterial effectors has revealed that their type III molecular syringe consists of a complex structure derived from the flagellum. Guy Cornelis (Biozentrum, Basel, Switzerland) presented a full three-dimensional structure of this complex and showed that it is composed of proteins with auto-assembly properties. This machinery allows specific bacterial proteins to be injected into plant cells through membranes and cell walls. Novel bacterial proteins injected through this mechanism were identified and included effectors with multiple functional protein domains, suggesting that there are a large number of possible targets in plant cells, as reported in a runner-up poster (poster 63: Plant proteins targeted by the GALA type III effector family from the pathogenic bacterium *Ralstonia solanacearum*. P. Remigi, I. Kars, S. Genin, C. Boucher and N. Peeters). Indeed, a wide range of plant proteins targeted by bacterial effectors were identified (kinase, NBS-LRR, ubiquitin ligase, RNA-binding protein and enzymes from the micro-RNA pathway). Additionally, Ulla Bonas (Martin-Luther-University Halle-Wittenberg, Germany) described how the effector, AvrBs3, acts on DNA by binding to plant promoters and functioning as a transcriptional activator. AvrBs3 was discovered to bind DNA through a unique mechanism, following a code for sequence recognition that depends on the number of repeats of the protein and on the sequence of two hypervariable residues (Boch *et al.*, 2009). Bacterial secondary metabolites were also shown to act as effectors, with Syringolin A acting as a proteasome inhibitor, as reported in a runner-up poster (poster 50: The small effector molecule Syringolin A inhibits the plant proteasome *in vivo* and during infection. J. J. Misas-Villamil, I. Kolodziejek, J. Clerc, M. Kaiser, M. Verdoes, H. Overkleeft, B. Schellenberg, R. Dudler and R. A. L. van der Hoorn). The evolutionary genomics of bacterial effectors was also boosted by the increased number of available bacterial genomes, revealing a high genetic diversity within effector families from related strains/species (Stephan Genin, LIPM, Toulouse, France; Brian Stackawicz, University of California-Berkeley, USA).

Jim Alfano (University of Nebraska, USA) reported how large-scale *in planta* functional screens have revealed that a large number of *Pseudomonas syringae* type III effectors inhibit plant defenses (Guo *et al.*, 2009). Greg Martin (Cornell University, NY, USA) discussed AvrPtoB, another

cell death-suppressing type III effector of *P. syringae*. AvrPtoB targets the tomato protein Bti9, a receptor-like protein kinase orthologous to the *Arabidopsis thaliana* chitin receptor Cerk1. Hairpin silencing of Bti9 in tomato enhanced susceptibility to bacteria. AvrPtoB was previously known to interact with the resistance protein Pto. Martin suggested that Pto may have evolved as a decoy of Bti9, the potential operative target of AvrPtoB (Van der Hoorn & Kamoun, 2008).

Translocation of eukaryotic microbe effectors into plant cells: towards conserved mechanisms

In eukaryotic pathogens, effectors are secreted in plant tissues through classical secretion machineries. These effectors can diffuse in the plant apoplast or enter into plant cells through largely unknown mechanisms. Several presentations have illustrated how research on effectors from oomycetes has brought some clarity to this topic (Dodds *et al.*, 2009). Indeed, genome-wide analyses of these eukaryotic filamentous organisms related to brown algae have shown that they carry a large number of gene families encoding candidate effectors with either the RxLR-dEER motif (~550 in the potato blight pathogen *Phytophthora infestans*) or the LFLAK motif (Crinkler family ~200 members in *P. infestans*) (Haas *et al.*, 2009). Sophien Kamoun (The Sainsbury Laboratory, UK) reported that the Crinkler effectors have a wider species distribution (Peronosporales to Saprolegniales) than the RxLR family (Peronosporales). Expansion of these multigenic families was observed in all plant pathogenic species including, as reported by Jim Beynon (University of Warwick, UK), *Hyaloperonospora arabidopsidis*, a biotrophic oomycete pathogen. Brett Tyler (Virginia Bioinformatics Institute, USA) reported significant progress on the mechanisms of translocation of RxLR effectors into plant cells. Experimental evidence was presented for the rapid and efficient uptake of RxLR proteins into plant and human cells through a phosphatidyl-inositide phosphate-facilitated process. This process requires the RxLR-dEER motif that is located after the signal peptide (the N-term of the mature protein) and that resembles the PEXEL translocation motif of *Plasmodium* effectors. Significant progress was also made in understanding the biochemical activities of RxLR effectors. Paul Birch (Scottish Crop Research Institute, UK) reported that *P. infestans AVR3a* targets CMPG1, an E3 plant ligase, as well as Sec5, a plant protein involved in exocytosis.

Translocation into plant cells of effectors from plant pathogenic fungi was also shown in several cases, namely Pwl2 from *Magnaporthe oryzae*, AvrL567 from *Melamspora lini* and ToxA from *Pyrenophora tritici*. According to Brett Tyler (Virginia Bioinformatics Institute, USA), some fungal effectors carry degenerate RxLR-like motifs. When tested using a soybean root-translocation assay designed for oomycete effectors, RxLR motifs from three fungal effectors (AvrL567, Six1 and AvrLm6) proved sufficient to translocate green fluorescent protein (GFP) into plant cells. These results enable the identification of candidate effectors and thus potentially open up a whole new research area in fungal effectomics. These findings also imply that different pathogens use similar strategies to deliver their proteins into host cells. Genome-wide analysis of fungal effectors has also revealed either their biased localization in transposon-based AT-rich regions of the *Leptosphaeria maculans* genome, as discussed by Thierry Rouxel (BIOGER-INRA, Grignon, France), or their expansion in the *Cladosporium fulvum* genome (Pierre de Wit, Wageningen University, the Netherlands). Pierre de Wit also reported biochemical activities, such as binding to chitin and inhibition of proteases, of some *C. fulvum* apoplastic effectors (De Wit *et al.*, 2009). Also, as reported for bacteria, fungal secondary metabolites were shown to act as effectors, either as toxins facilitating infection or as avirulence signals recognized by resistant plants (Marc-Henri Lebrun, BIOGER-INRA, Grignon, France).

Do effectors from symbiotic bacteria and fungi play a role in these interactions?

The genomes of plant symbiotic bacteria also contain a small number of effector-encoding genes injected through a type III molecular syringe, as reported by William Deakin (Université de Genève, Switzerland). These effectors have either positive or negative effects on symbiosis. Indeed, the same bacterial effector was shown to act positively on symbiosis during the interaction with *Medicago truncatula*, while it was detrimental during the interaction with *Phaseolus vulgaris*, making the overall response more complex. Francis Martin (INRA-Nancy, France) described how the genomes of symbiotic fungi also carry genes encoding effectors, as observed in the ectomycorrhizal symbiont *Laccaria bicolor* (Martin *et al.*, 2008). Genome analysis of *L. bicolor* revealed a large number of genes encoding small secreted proteins that were overexpressed during symbiosis. The most highly expressed effector during symbiosis (MiSSP7) specifically accumulates in fungal cells colonizing the root apoplasm. Silencing of this gene dramatically reduced fungal root colonization, demonstrating, for the first time, that a fungal effector is required for symbiosis. *Glomus intraradices*, an endomycorrhizal symbiotic fungus, also produces effectors that are secreted and translocated into the root cell nucleus, as illustrated in the talk of Natalia Requena (University of Karlsruhe, Germany). Expression of this protein in plant root facilitated the establishment of symbiosis. These results were also presented in a poster that received the First Prize during this meeting (poster 37: A putative novel effector family in arbuscular mycorrhizal fungi. S. Kloppholz and N. Requena). Overall, these presentations clearly demonstrate

that fungal symbionts use proteins secreted in host plant tissues to interact with their hosts, extending the importance of effectors from pathogens to beneficial microbes.

Effectors in plant insect–nematode interactions: an emerging field

Effectors are not limited to microbial pathogens as they have also been identified in insects and worms. Richard Hussey (North Carolina State University, USA) described how plant-associated nematodes inject peptides into host plant cells that act on plant transcription or that mimic plant-signaling peptides, such as Clavata 3 (Davis *et al.*, 2008). A genome-wide survey of nematode genome sequences confirmed the presence of gene families encoding a variety of candidate effector proteins. Gerald Reeck (Kansas State University, USA) reported that insects, such as aphids, also secrete effectors into their saliva, allowing them to be injected into plant cells when the insect pricks host tissues with its stylus (Mutti *et al.*, 2008).

Conclusions

The study of effectors is an emerging and unifying theme across plant-associated microbes. The symposium strengthened the view that effectors play a pivotal role in plant–microbe interactions. Indeed, both symbiotic and pathogenic microbes appear to mobilize a rich assortment of effectors to manipulate host plant cells. The symposium was useful in bringing together researchers who have been studying effectors for a long time and have reached the point of detailed biochemistry, with other researchers who have only recently discovered effectors in the genome of their favourite plant-associated microbe. One take-home message of this meeting was the fact that the concept of effectors has clearly expanded to symbionts and plant-associated nematodes and insects. This promises a future rich in exciting discoveries and insights into plant–microbe interactions.

Acknowledgements

We would like to thank Holly Slater, Helen Pinfield-Wells and Jill Brooke from *New Phytologist*, as well as Anne-Sophie Simon and members of BIOGER for their tremendous input in the organization of this meeting.

Marc-Henri Lebrun[1]* and Sophien Kamoun[2]

[1]BIOGER, UMR INRA-APT, Campus Agroparistech, 78850 Thiverval Grignon, France; [2]The Sainsbury Laboratory, Colney Lane, Norwich, NR4 7UH, UK
(*Author for correspondence:
email marc-henri.lebrun@bayercropscience.com)

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Key words: bioaggressors, effectors, genomics, pathogens, plant–insect interactions, plant–microbe interactions, symbionts, type III secretion.

Soil respiration across scales: towards an integration of patterns and processes

European Science Foundation Exploratory Workshop 'Diurnal- to century-scale controls on soil respiratory fluxes. Towards a new generation of integrated experimental and modelling approaches', Innsbruck, Austria, September 2009

In view of a rapidly changing climate system there has been a growing interest in the role of ecosystems in the global carbon (C) cycle. Considerable uncertainties still exist concern-

ing the factors determining soil respiration (SR), the largest source of CO_2 from terrestrial ecosystems, and how it will respond and feed back to climate change. Soil respiration includes a component derived from C recently assimilated by plants (i.e. through the respiration of roots, mycorrhizas and rhizosphere microorganisms) and a component derived from the C respired during the decomposition of dead plant litter, microbial debris and destabilized soil organic matter (SOM) (Fig. 1). Thanks to a range of observational and manipulative studies, and to advances in technology, progress has been made in our understanding of biotic and abiotic effects on soil C fluxes (Kutsch *et al.*, 2009; Norby, 2009; Paterson *et al.*, 2009). The European Science Foundation Exploratory Workshop addressed the need to synthesize recent insights on diurnal- to century-scale controls of soil respiratory fluxes and to incorporate them into a new generation of process-based models, for developing a larger framework with an improved capacity for predicting SR in changing environments.

'…The ultimate goal for model development must be to overcome "dead-soil-box paradigm" models, that is, to consider key processes and properties governing soil C dynamics, including vertical differentiation and transport mediated by roots and soil biota, and root–microbial as well as microbial–mineral interactions with respective effects on C dynamics.'

Short-term links between photosynthesis and soil respiration

In recent years there has been increasing evidence that plant activity is a key determinant of SR (e.g. Högberg & Read, 2006). Besides producing litter as a major substrate for heterotrophic activity in the soil, plants directly influence soil respiratory processes via root respiration and by providing photo-assimilates that fuel the metabolic processes of mycorrhizae, endophytes and microbial populations in the rhizosphere (Fig. 1). Easily degradable compounds, including root exudates, may also provide energy for stimulating an increased microbial breakdown of more complex SOM compounds, the so-called priming effects of SOM decomposition (Kuzyakov, 2002) (Fig. 1).

While the effects of photosynthesis on SR have been well documented at annual and seasonal timescales, a consistent picture of their short-term coupling is only just beginning to emerge. Yakov Kuzyakov (University of Bayreuth, Germany) presented a synthesis of a range of short-term studies indicating that a photosynthetic signal appears in soil-respired CO_2 within hours (herbaceous vegetation) to a few days (trees) (Kuzyakov & Gavrichkova, 2010). While isotope tracer studies track the time for transfer of individual C atoms from the canopy to the rhizosphere, photosynthetic signals may theoretically be

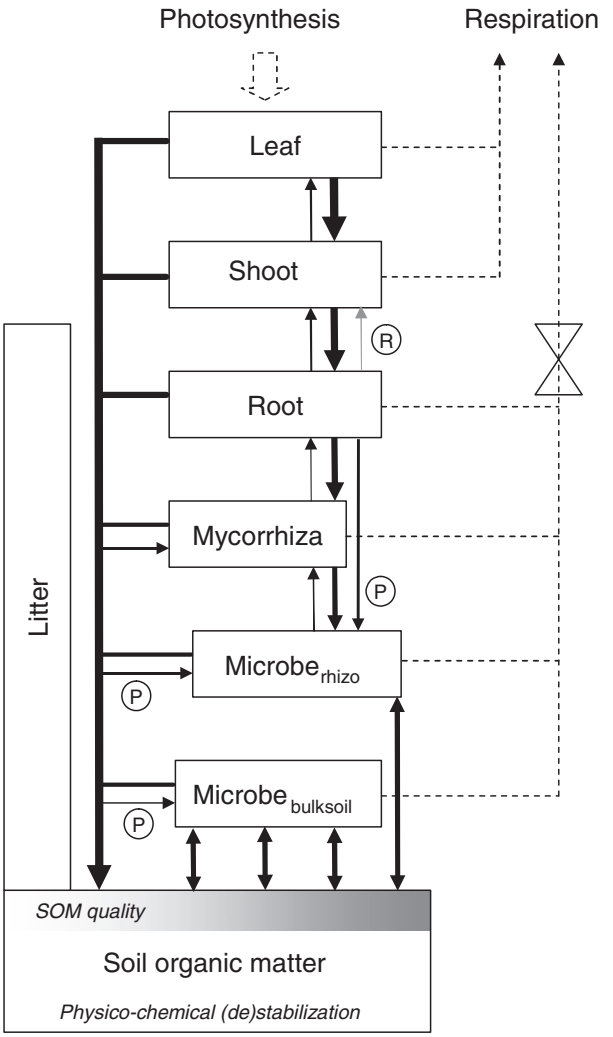

Fig. 1 Major carbon (C) pools (boxes) and fluxes (arrows) in the plant–soil system, including plant, mycorrhizal, rhizosphere and bulk soil microbial communities. Gaseous fluxes are indicated by broken lines. **P,** potential priming effects; **R,** the transport of refixed root-respired CO_2 to above-ground plant parts. ⧖ symbolizes the effects of gas diffusivity in the soil pore space on the transfer of respired CO_2 to the soil surface. Shades in the soil organic matter (SOM) box indicate a gradient of complexity and of the molecular weight of organic C compounds. For simplicity, faunal components grazing on all aboveground and belowground C pools and feeding into the litter and the SOM pools have not been included. For more detailed depictions see Kutsch *et al.* (2009) and Paterson *et al.* (2009).

transmitted much more rapidly to the soil by pressure-concentration waves (Mencuccini & Hölttä, 2010). Jorge Curiel Yuste (University of Barcelona, Spain) and Rodrigo Vargas (University of California, Berkeley, USA) provided two synthetic studies that explored the relationships between canopy photosynthesis (as derived from eddy covariance measurements) and SR, based on continuous measurements in different vegetation types across the growing season. These larger-scale studies only partly confirm a strong and rapid coupling between photosynthesis and SR. They suggest that there might be a fast link between these processes at the timescale of hours and a slower link at the timescale of a few days, while there may also be extended periods in the season without any clear correlation between photosynthesis and SR.

Short-term relationships between photosynthesis and SR may be obscured by low or changing soil diffusivity, which affects the transfer rates from CO_2 produced in the soil to the soil surface (Stoy *et al.*, 2007) (Fig. 1). Furthermore, as suggested by isotopic labelling and shading experiments, the involvement of different substrate pools within the plant may lead to a temporary decoupling between photosynthesis and SR, with storage pools buffering short-term changes in photosynthetic C uptake (Carbone & Trumbore, 2007; Bahn *et al.*, 2009). Susan Trumbore (University of California, Irvine, USA) and Mariah Carbone (University of California, Santa Barbara, USA) presented further evidence, based on radiocarbon analyses, that CO_2 from root respiration is only partly derived from very recently fixed C pools. Moreover, Arthur Gessler (University of Freiburg, Germany) demonstrated that a refixation of root-respired CO_2 and its upward export to aboveground plant tissues (Aubrey & Teskey, 2009) can cause a mixing of different substrate pools, for example in the trunks of trees (Kodama *et al.*, 2008), and may potentially also lead to an underestimation of the amount of CO_2 respired belowground, as determined using soil surface CO_2 efflux measurements (Fig. 1). Finally, irrespective of the size and age of plant C pools, during periods of increased nutrient demand by plants, rhizodeposition is often increased, stimulating SOM decomposition in the rhizosphere (Frank & Groffman, 2009; Jones *et al.*, 2009).

C fluxes in the plant–soil system: differential responses and effects of different biota

Different components of the soil system may access and use fresh photoassimilates to different degrees. Peter Högberg (Swedish University of Agricultural Sciences, Sweden) demonstrated, for a boreal forest, that fresh plant-derived C entering the soil is transferred mainly to fungal components, and less to bacterial components, and is taken up to very different degrees by different faunal groups. Andreas Heinemeyer (University of York, UK) showed that excess C from photosynthesis may affect mycorrhizal respiration more immediately and strongly than root respiration. Sébastien Fontaine (INRA, France) provided further evidence for the hypothesis that two distinct microbial functional groups respond differently to priming by fresh plant C inputs vs increased nitrogen (N) availability. Slow-growing, 'mining' microbes are more responsive to fresh plant C inputs, while fast-growing, 'storing' microbes are affected more by changes in N availability (Fontaine & Barot, 2005). He suggested that their interactions exert a major control on SOM dynamics. Furthermore, biotic interactions involving different trophic levels, such as aboveground and belowground herbivory, may alter source–sink relations and thus C fluxes in the plant–soil system, thereby substantially affecting soil C dynamics in changing environments (Ayres *et al.*, 2009).

Modelling soil respiration and soil C turnover

To be able to account for multiple processes operating at diurnal to annual timescales, process-based models of SR should take into account more than simple soil temperature and moisture relationships. Markus Reichstein (Max Planck Institute for Biogeochemistry, Germany) showed that ignorance of plant–soil coupling leads to erroneous interpretations of respiration in relation to abiotic drivers in models (e.g. to an overestimation of the temperature sensitivity) (Reichstein & Beer, 2008). Data analysis (and modelling) on explicitly distinct timescales offers new perspectives for inferring processes and disentangling confounded responses to abiotic and the above-mentioned abiotic drivers (Mahecha *et al.*, 2010). The ultimate goal for model development must be to overcome 'dead-soil-box paradigm' models, that is, to consider key processes and properties governing soil C dynamics, including vertical differentiation and transport mediated by roots and soil biota, and root–microbial as well as microbial–mineral interactions with respective effects on C dynamics (e.g. priming).

Eric Davidson (Woods Hole Research Center, USA) pointed out that most available soil C turnover models have been successful in describing the effects of land use, but may be less suitable for assessing the impacts of climate change. Developing further the perspective outlined by Davidson & Janssens (2006), he suggested that this limitation could be overcome by separating physico-chemical processes (aggregation, sorption-desorption, hydrophobicity) from enzymatic ones. He furthermore emphasized that SOM pools should not be based on age, but on process. Such a new generation of models would ideally be structured in a modular way, and would include modules on plant C inputs, microbiology, SOM quality (distinguishing assimilable from higher-molecular-weight compounds) and soil physics. Pete Smith (University of Aberdeen, UK) highlighted recent developments in soil C-fractionation schemes

that allow measurable and modelled pools to be reconciled (Zimmermann *et al.*, 2007), which could help to bridge the gap between experimentalists and modellers, and contribute to improved model-validation schemes.

Conclusion and outlook: towards a new generation of integrated experimental and modelling approaches

In the presentations and many discussions at the workshop, a number of issues emerged that need to be addressed in more detail in the future: (1) the plant-to-soil C transfer, as related to plant C allocation and belowground C partitioning, focusing on the interactions with the N cycle, soil and vegetation types, and phenology, and as affected by changing environmental conditions; (2) soil C turnover, with particular reference to the effects of substrate quality and quantity on decomposition rates, and the mechanisms of soil C stabilization and destabilization (aggregates, priming) across soil and vegetation types, as affected by the quantity and quality of soil C inputs (litter, exudates, dissolved organic and inorganic C); (3) the role of biota for SOM pools and fluxes, considering the effects of different functional groups (e.g. fungi, bacteria, soil fauna); and (4) the development of soil C models, particularly for improving suitability for climate change scenarios, separating physico-chemical from enzymatic processes and improving the representation of different SOM pools. Such models should advance the description of physico-chemical stabilization and the interface between ecophysiology (C allocation) and soil modules and account for vertical soil heterogeneity and biotic interactions. The explicit incorporation of ^{13}C and ^{14}C will permit the use of isotopic data sets for model testing and validation.

From the insights outlined above it is clear that future research efforts on soil C dynamics would profit substantially from increased collaboration among plant ecophysiologists, microbiologists and soil scientists. Linking experimental and advanced model data-integration approaches will help to develop, refine and test a consistent framework for understanding and predicting soil respiratory fluxes across spatial and temporal scales.

Acknowledgements

Apart from the experts highlighted above, the following workshop participants presented their work and contributed substantially to the intense discussions: Werner Borken, Alf Ekblad, Daniel Epron, Werner Eugster, Werner Kutsch, Jari Liski, Bernard Longdoz, Laurent Misson, Jerome Ngao, Marian Pavelka, Jukka Pumpanen, Andreas Richter, Mirco Rodeghiero, Jens Subke, Lisa Wingate and Thomas Wutzler. Pete Smith is a Royal Society-Wolfson Research Merit Award holder. The Exploratory Workshop was funded by the European Science Foundation (EW08–057). Further financial support was provided by the University of Innsbruck.

Michael Bahn[1]*, Ivan A. Janssens[2], Markus Reichstein[3], Pete Smith[4] and Susan E. Trumbore[3]

[1]Institute of Ecology, University of Innsbruck, Sternwartestr. 15, 6020 Innsbruck, Austria; [2]Department of Biology, University Instelling, Antwerp, B-2610 Wilrijk, Belgium; [3]Max-Planck Institute for Biogeochemistry, Hans-Knöll-Str. 10, 07745 Jena, Germany; [4]Institute of Biological and Environmental Sciences, School of Biological Sciences, University of Aberdeen, Aberdeen AB24 3UU, UK
(*Author for correspondence: tel +43 512 5075905; email michael.bahn@uibk.ac.at)

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Key words: carbon allocation, carbon cycle, model–data integration, photosynthesis, rhizosphere, soil biota, soil CO_2 efflux, soil organic matter decomposition.

Obituary

Image courtesy of Przemyslaw Ryszka, Jagiellonian University, Krakow, Poland

Prof. Dr Gopi Krishna Podila, 1957–2010

The sudden death of Gopi Krishna Podila was a staggering blow to his immediate colleagues and to all those who knew this wise, amiable man with a great smile and infectious laugh. The world of plant and microbial sciences has lost one of its leading figures and a most respected champion of mycorrhizal biology and genetics. As such, he was an active member of the Advisory board to *New Phytologist* and an Editor of *Symbiosis* and *Journal of Plant Interactions*. That Gopi has been taken from us whilst at the height of a productive career is tragic, and particularly so given that he died alongside two colleagues after a shooting at the University of Alabama (Huntsville). He is survived by his wife and two daughters, his mother and his three brothers.

Gopi was born in 1957 at Guntur, Andhra Pradesh, India. He initiated his academic career at the Nagarjuna University, India, where he obtained training in the fields of biology, plant pathology and soil microbiology at both undergraduate and postgraduate levels. Gopi followed this with a move to the USA, where he studied plant pathology at Louisiana State University, ultimately graduating with a Masters degree. He continued his graduate education at Indiana State University where he obtained a PhD in Molecular Biology in 1987 before undertaking a postdoctoral fellowship at the Ohio State University under the guidance of Dr Pappachan Kolattukudy. Gopi joined the Department of Biological Sciences at Michigan Technological University as Assistant Professor in 1990 and rose through faculty ranks to become the Professor and Adjunct Associate Professor in the School of Forestry within a short period of time. In 2002 Gopi moved to the University of Alabama in Huntsville, where he had been appointed to lead the Department of Biological Sciences both as Professor and Chair. In this dual role, Gopi was instrumental in strengthening both the teaching program in Huntsville as well as developing links with the broader biology community in the area, in particular the biotechnology industry. To top his scientific achievements, he was an excellent teacher, a trait he demonstrated with great enthusiasm while interacting with students of all levels, from high school through undergraduate to doctoral levels. Never to raise his voice, he was a patient, amicable and polite teacher and coworker.

A brief review of his work, which led to so many insights, shows that, above all, he had the knack of seeing how new

molecular techniques could be used to decipher complex signalling mechanisms of plant–microbe interactions. A series of landmark papers published in *Science*, *Proceedings of the National Academy of Sciences (USA)* and *Nature* with several colleagues in Dr Kolattukudy's laboratory, concerned the rather puzzling signalling pathways between the phytopathogenic fungus *Fusarium solani pisi* and its host plant. These early works set the stage for a lifelong interest in how fungi interact with plants. At Michigan Technological University, his group identified several symbiosis-related genes regulated during ectomycorrhizal symbiosis development.

His laboratory was one of the first to genetically engineer mycorrhizal fungi for functional genomic studies. As an expert in symbiosis research, Gopi is perhaps now chiefly remembered for his contributions to our understanding of the mechanics of signalling that lead to ectomycorrhizal development, many of his papers having been published in *New Phytologist*. Gopi's efforts were instrumental to the success of the project leading to the sequencing of the genome of the ectomycorrhizal fungus *Laccaria bicolor*, the first, and only, mycorrhizal fungus genome to be sequenced to date. Only a few days before his tragic death, he was discussing how to use RNA-Seq data to improve the most recent annotation of this genome. There were, however, many other areas, such as poplar genomics, in which he laid a firm foundation on which later work could thrive. As an *aficionado* of symbiotic systems, Gopi was a key member of the International Society for Symbiosis: he was a Governing Councilor of the Society for over 10 yr, and was among the most active editors of its journal *Symbiosis* since 2000. Present at all meetings of the Society, he contributed very often to the creation of cross-disciplinary interactions and to links between the different models.

To those who were fortunate enough to know him, his death leaves an empty space. He would listen attentively, with his head slightly tilted to one side and a twinkle in his eye, and then he would ask a question which would go straight to the heart of the matter. He was never harsh or malicious. His criticisms were kindly and his suggestions invariably constructive. An ardent lover of music, and a researcher to the core with an eye to catch the minutest detail, he was an excellent colleague and friend who shall be missed dearly; we will all remember his periodic emails of good wishes and seasonal greetings, and his infectious laugh, which enlightened our days no matter where we were in the world.

Francis Martin & Marc-André Selosse
Interaction Editors, *New Phytologist*