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Plant Defense: a Pre-adaptation for Pollinator Shifts

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Commentary

Pigment loss in response to the environment: a new role for the WD/bHLH/MYB anthocyanin regulatory complex

The bright reds, blues and purples produced in most plants by the anthocyanin biosynthetic pathway have been of perennial fascination to plant biologists. Being conspicuously colorful, dispensable and tractable have made these flavonoid-based pigments ideal subjects for studying a variety of biological phenomena in a number of plant models. Indeed, seed coat and flower color of peas were among the phenotypes used by Gregor Mendel in his studies of inheritance, and Barbara McClintock followed curious color changes in maize kernels in her discovery of transposable elements. More recently, in petunia, anthocyanin loss as a result of the overexpression of flavonoid biosynthetic genes, first noticed by Richard Jorgensen and colleagues, helped to define the phenomenon of cosuppression (RNA interference or RNA mediated gene silencing) (Napoli *et al.*, 1990; Jorgensen, 1995). A significant part of this rich research tradition is a wealth of work on transcriptional regulation in plants for which the anthocyanin pathway has been an excellent model. In fact, the first plant transcription factor cloned was *Colorless1* (*C1*) (Cone *et al.*, 1986), a maize MYB regulator of the flavonoid pigment biosynthetic pathway. From this, and from subsequent work in a host of plant species, emerged the now canonical WD-repeat/bHLH/MYB combinatorial transcription factor complex model for the regulation of the anthocyanin pathway (Goff *et al.*, 1992; Spelt *et al.*, 2000; Zhang *et al.*, 2003; Schwinn *et al.*, 2006; Gonzalez *et al.*, 2008). The story in *Arabidopsis* is particularly interesting as there are four R2R3 MYB activators (PAP1, PAP2, MYB113 and MYB114), three bHLH activators (TT8, EGL3 and GL3) and at least one R3 repressor (MYBL2) that can physically interact with each other to form various transcriptional complexes with the WD-repeat protein, TTG1 (Gonzalez *et al.*, 2008; Matsui *et al.*, 2008). This suggests a combinatorial transcription factor mechanism behind the fine regulation of pigment expression observed developmentally and in response to various biotic and abiotic stresses. Indeed, we have begun to elucidate the molecular mechanisms of how the *Arabidopsis* regulators differentially contribute to pigment production in response to specific stimuli that promote pigment production (Teng *et al.*, 2005; Cominelli *et al.*, 2007; Lea *et al.*, 2007). While much is known about how anthocyanin production is positively regulated in response to various conditions, little is known about the mechanisms responsible for anthocyanin catabolism in response to certain environmental stimuli. In this issue of *New Phytologist*, Rowan and colleagues (pp. 102–115) provide new insights that enhance our understanding of anthocyanin pathway regulation by the TTG1/bHLH/MYB complex in response to conditions that promote anthocyanin turnover and degradation. This work is unique in its approach; not only examining anthocyanin turnover and degradation in *Arabidopsis* as a response to environmental conditions promoting pigment loss, but also establishing a novel connection to the *Arabidopsis* TTG1/bHLH/MYB complex. This is in refreshing contrast to numerous studies over the years emphasizing primarily the production of pigments through the positive regulation of anthocyanin biosynthesis by the TTG1-dependent transcriptional complex.

'The work of Rowan et al. *defines a new role for the WD/bHLH/MYB anthocyanin regulatory complex by showing that it mediates the response to environmental stimuli which promote pigment loss.'*

New twists on old favorites

The *PAP1* MYB regulator of the anthocyanin pathway was first identified in striking fashion by an activation tag screen; the *Arabidopsis* mutant overexpressing this MYB appears to be deeply pigmented over much of the plant body and life cycle (Borevitz *et al.*, 2000). This was apparently a result of the upregulation of not just a subset of anthocyanin structural genes, but genes across the entire general phenylpropanoid pathway (Borevitz *et al.*, 2000; Tohge *et al*, 2005). Seemingly broad regulation of phenylpropanoid metabolism, coupled with a dramatic gain-of-function phenotype, made the *PAP1* overexpressor mutant a fascinating research subject with possibly much to tell about transcriptional control invested in a single transcription factor over a considerable swath of metabolic pathway. While the subsequent emphasis on what *PAP1* can do when overexpressed proved fruitful in identifying new

anthocyanin pathway genes (Tohge *et al.*, 2005), questions lingered about the limitations of this MYB, about native pathway regulation by *PAP1* and about the contributions to anthocyanin pathway regulation by the closely related paralogous MYBs, namely *PAP2*, *MYB113* and *MYB114*. Some of these questions have only recently been addressed, again with the help of PAP1 overexpressor mutants (Teng *et al.*, 2005; Cominelli *et al.*, 2007; Lea *et al.*, 2007; Gonzalez *et al.*, 2008).

However, the work of Rowan and colleagues presents a clever twist on the use of the PAP1 overexpressor. These authors identify an environmental condition (high temperature, low light; HTLL) that results in the reversible loss of pigmentation despite the overexpression of PAP1. In-depth profiling of anthocyanin content and biosynthetic gene expression indicates that pigment loss caused by HTLL is a function of both anthocyanin turnover and downregulation of the flavonoid pathway, consistent with previous work in grape and rose (Dela *et al.*, 2003; Mori *et al.*, 2007). But, unlike previous studies, new mechanisms are proposed that account for the downregulation of the anthocyanin pathway and loss of pigments in plants under HTLL. First, other members of the anthocyanin-regulatory complex, such as *EGL3* and *TT8* bHLH genes, and *TTG1* encoding the WD-repeat protein, are downregulated by HTLL treatment. Second, the gene encoding the R3 repressor MYB (MYBL2), which competes with R2R3 activator MYBs for binding to bHLH partners, is upregulated by HTLL treatment. This, in turn, would explain decreases in the expression of anthocyanin structural genes, even when PAP1 is overexpressed. This is an important finding with respect to the long-studied WD-repeat/bHLH/MYB anthocyanin regulatory complex; it demonstrates that the complex itself is robustly targeted (by the downregulation of activator members and by the upregulation of repressor members) as a novel mechanism mediating the response of anthocyanin loss to abiotic conditions.

Another interesting mechanism possibly contributing to the loss of anthocyanins under HTLL treatment is suggested by the data of Rowan *et al*. Various steps in the pigment biosynthetic pathway that are upregulated by PAP1 are observed to be downregulated throughout the duration of HTLL treatment (consistent with the loss of pigment). A notable exception, however, was the expression pattern of the glutathione *S*-transferase gene, *TT19*. This known target of the transcriptional complex is necessary for vacuolar accumulation of anthocyanins in *Arabidopsis* but is only transiently downregulated during HTLL treatment. Later upregulation of *TT19* during HTLL treatment suggests that a high rate of transport of simple anthocyanin intermediates into the vacuole limits the availability of these intermediates for pigment production. Pigment loss would then result from anthocyanin degradation in the absence of new synthesis.

Future research: what regulates the regulators?

The work of Rowan *et al*. defines a new role for the WD/bHLH/MYB anthocyanin regulatory complex by showing that it mediates the response to environmental stimuli which promote pigment loss. By altering the composition of the transcriptional complex in response to HTLL, regulation of anthocyanin pathway genes is achieved. However, the questions remain of what links the expression changes of regulatory genes, such as *TT8* and *EGL3*, to the environment; and what connects the WD/bHLH/MYB complex particularly to abiotic conditions that cause anthocyanin loss? Despite a research history of MYB and bHLH anthocyanin regulators spanning two decades, virtually nothing is known about what directly influences members of this heavily studied complex in response to biotic and abiotic stimuli. Hormones have been implicated in influencing the expression or function of the complex but, again, it is not known how many steps removed this regulation might be from direct control of the regulators. Research efforts that move upstream of the WD/bHLH/MYB complex are warranted and should reveal new mechanisms that help translate environmental stimuli to control of the flavonoid biosynthetic pathway.

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Key words: abiotic response, anthocyanins, Arabidopsis, bHLH, MYB, PAP1.

Chlorophyll and folate: intimate link revealed by drug treatment

Chlorophyll biosynthesis is one of the major biosynthetic pathways in nature. A staggering 10^9 tonnes of chlorophyll are synthesized and degraded by the earth's vegetation each year, the only biological process that can be visualized from space (Rudiger, 1997). Because of the importance of chlorophyll biosynthesis, it has been studied extensively at both molecular and biochemical levels. To synthesize the tetrapyrrole macrocycle of chlorophyll *a*, just three precursors are needed: eight molecules of glutamate, a Mg^{2+} ion and a methyl group. This is then linked to a phytol chain that is synthesized via the isoprenoid pathway. Studies on the relationship between the isoprenoid and tetrapyrrole pathways have found that they are closely linked, so that inhibition of one pathway affects the flux through the other (Laule *et al.*, 2003). In more recent years, attention has focussed on the co-ordination between the production of chlorophyll and that of the chlorophyll-binding proteins, most of which are encoded by the nucleus (reviewed in Tanaka & Tanaka, 2007). Until now, however, there has been little consideration of how the pathway integrates with C1 metabolism for the supply of the methyl group, required for the conversion of Mg-protoporphyrin IX to Mg-protoporphyrin IX methyl ester by Mg-protoporphyrin IX methyltransferase (Fig. 1). The methyl group is derived from *S*-adenosylmethionine (SAM), which in turn obtains its methyl group from the folate pool. Now, van Wilder *et al*. have, for the first time, demonstrated a link between chlorophyll biosynthesis and folate (this issue, pp. 137–145).

Their experimental system was the de-etiolation of pea seedlings, in which there is rapid production of new chlorophyll. Pretreatment of the seedlings with methotrexate, an inhibitor of folate metabolism, caused a reduction in the rate of chlorophyll production, so that after 24 h of illumination there was less than half the chlorophyll found in untreated leaves. In the methotrexate-treated tissue there was a slight reduction in total folate, but a sevenfold reduction in the methylation index (the ratio of the concentrations of SAM to *S*-adenosylhomocysteine (SAH)). At the same time, Mg-protoporphyrin IX methyltransferase activity was reduced threefold.

'... the distribution of folate species in the cell can have dramatic effects on the rate of chlorophyll biosynthesis.'

Methotrexate is a chemotherapeutic agent, used in cancer therapy, and the mechanism of action of this drug in human cells, namely the inhibition of dihydrofolate reductase (DHFR; Fig. 1), is well characterized (Huennekens, 1994). Treatment with methotrexate leads to depletion of methylene tetrahydrofolate as a substrate for thymidylate synthase (TS) and to depletion of 10-formyl tetrahydrofolate, the substrate for two enzymes of *de novo* purine biosynthesis, both of which impair

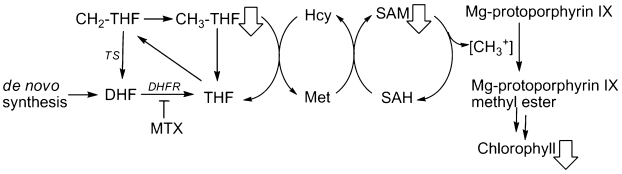

Fig. 1 Relationship among folate, C1 metabolism and biosynthesis of chlorophyll in de-etiolating pea seedlings. Treatment of etiolated pea seedlings with methotrexate (MTX) inhibits dihydrofolate reductase (DHFR), causing a subsequent reduction in the cellular concentration of methyl tetrahydrofolate (CH_3-THF). This, in turn, causes depletion in the concentration of *S*-adenosylmethionine (SAM), so there is a concomitant reduction in chlorophyll production during de-etiolation. CH_2-THF, methylene tetrahydrofolate; Hcy, homocysteine; Met, methionine; SAH, *S*-adenosylhomocysteine.

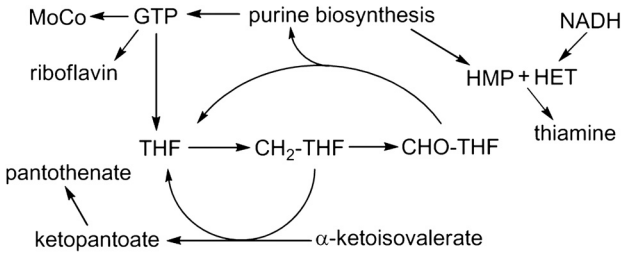

Fig. 2 Interconnections between vitamin biosynthetic pathways in plants. CHO-THF, *N*-formyl tetrahydrofolate; HET, hydroxyethylthiazole; HMP, hydroxypyrimidine; MoCo, molybdenum cofactor.

DNA synthesis. This, in turn, has pleiotropic effects, leading to reduced cell growth and eventual apoptosis. van Wilder *et al.* consider methotrexate inhibition of DHFR in the context of the *de novo* biosynthetic pathway of folate (i.e. that inhibition of the enzyme leads to a reduced flux through the biosynthetic pathway). It is conceivable that in pea seedlings, as in human cells, methotrexate also inhibits recycling of dihydrofolate to tetrahydrofolate by TS, and this may account for the observed phenotype. It would be interesting to determine the relative proportion of these forms of folate in the treated seedlings, particularly in the light of the fact that pea DHFR and TS have been shown to reside in the same polypeptide (Neuburger *et al.*, 1996). Nonetheless, this question does not detract from the main thesis of the paper: that the distribution of folate species in the cell can have dramatic effects on the rate of chlorophyll biosynthesis.

We should not be surprised by the observation of a link between chlorophyll and folate. In contrast to the amount of chlorophyll in the cell (in the order of 2 mg g^{-1} FW (Santana *et al.*, 1998)), only catalytic quantities of folate are present (~5 ng g^{-1} FW (van Wilder *et al.*)). This modest demand for folate is met by a very low flux through the biosynthetic pathway. However, like other vitamins and cofactors, folate is essential for plant cell metabolism (Smith *et al.*, 2007), and hence we should expect that small perturbations in its metabolism will have large pleiotropic effects. These effects may be even wider than at first thought because it has become apparent over recent years that the biosynthesis of these cofactors are closely interlinked with each other – the biosynthesis of one is often dependent upon the agency of another, and so reduction in the flux through one pathway may well lead to depletion of other cofactors. In the case of folates, there is a clear link to pantothenate metabolism, via the requirement for methylene tetrahydrofolate of the first committed enzyme, ketopantoate hydroxymethyltransferase (Fig. 2) (Ottenhof *et al.*, 2004). However, there are other, more distant, linkages. For example, 10-formyl tetrahydrofolate is required for the *de novo* biosynthesis of GTP. Three different cyclohydrolases cleave this molecule in the first committed steps of riboflavin (Bacher *et al.*, 2000), molybdenum cofactor (Mendel, 2007) and folate (Hanson & Gregory, 2002) biosynthesis. Other plant vitamin biosynthetic pathways also appear to be integrated: the redox cofactor NADH, which in plants is generated from aspartate and glyceraldehyde 3-phosphate, is converted to the thiazole moiety of thiamine by the action of THI1 and unknown accessory proteins (Godoi *et al.*, 2006), whilst the pyrimidine moiety is derived from aminoimidazolecarboxamide ribonucleotide (Raschke *et al.*, 2007) – an intermediate in purine biosynthesis.

Enzyme cofactors derived from vitamins such as folate, thiamine and riboflavin provide the chemistry for enzymatic reactions that cannot be carried out by amino acids. Consequently, their chemical nature is often complex, which is further reflected in the biosynthetic pathways (Smith *et al.*, 2007). This in turn makes their study intriguing. Moreover, because vitamins, by definition, are required in the human diet, there is interest in biotransformation systems for their synthesis, either to enhance levels in foodstuffs, or for commercial production of supplements. However, the low flux through plant vitamin biosynthesis pathways has complicated their study, so the identification of the enzymatic steps has proved challenging (Webb *et al.*, 2007). The connection between the regulation of a vitamin pathway and that of a major plant metabolite reported by van Wilder *et al.* is therefore even more exciting because it provides an avenue for the study of folate biosynthesis. It is likely in the future that a systems approach to studying plant metabolism will reveal many, as yet unrecognized, links between different pathways, shedding light onto their evolution on the one hand, and facilitating informed metabolic engineering on the other.

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Key words: chlorophyll biosynthesis, folate status, metabolic integration, methotrexate, vitamins and cofactors.

Plant defense: a pre-adaptation for pollinator shifts

Flowering plants are the most edible of all plants and yet the most successful. Darwin's 'abominable mystery' – the diversification of angiosperms – has often been attributed to their edibility and the subsequent evolution of herbivore defenses (Ehrlich & Raven, 1964; Frame, 2003). The origin of the most defining characteristic of angiosperms, the carpel, is widely believed to have evolved in order to protect the ovule from herbivory. While plant defense from herbivory has been extensively studied and even linked to angiosperm diversification (Ehrlich & Raven, 1964; Farrell *et al.*, 1992), the effects of herbivores on floral traits is rarely investigated (McCall & Irwin, 2006). In this issue of *New Phytologist*, Hanley *et al.* (pp. 251–260) describe a case where the evolution of plant defense pre-adapts lineages for pollinator shifts.

'... in Hakea, we see floral traits associated with plant defense evolve before pollinator-driven floral evolution, questioning the primacy of pollinators in floral evolution.'

Hanley *et al.* have revealed the evolutionary order of plant defense and pollinator shifts in the diversification of Western Australian *Hakea* spp. (Proteaceae) where bird pollination has evolved repeatedly. Transitions between insect and bird pollination are correlated with the origin of red flowers, longer stigma-to-nectary distances and increased inflorescence accessibility, which provide the perches necessary for Australian bird pollinators. Associated with these pollinator-driven changes in floral architecture are changes in plant-defense traits. Insect-pollinated Hakeas are physically defended from herbivores by dense foliage and spine-tipped leaves. The transition to bird pollination requires increased accessibility for birds and thus removes the plant's physical defenses, leaving the flowers open to florivory by emus and cockatoos. Hanley *et al.* have discovered an evolutionary correlation among floral cyanide levels (a chemical defense against avian florivory), bird pollination and associated floral traits. Across the phylogeny, the transition to bird pollination is contingent on the presence of elevated floral cyanide levels (Fig. 1c).

The primacy of pollinators?

In many lineages, pollinators are considered to be the fundamental drivers of floral diversity: flower color in *Mimulus* (Bradshaw & Schemske, 2003), spur length in *Aquilegia* (Whittall & Hodges, 2007) and pollen dosing in *Penstemon* (Wilson *et al.*, 2007), to name a few. Although pollinators are often deemed as the primary selective agents on floral morphology, nonpollinator agents may also be acting on flowers or be responsible for the origin of floral traits that are then co-opted during pollinator-mediated selection. Take, for example, the most common floral pigments, anthocyanins, which are omnipresent in angiosperms and probably evolved in early land plants long before the evolution of flowers. These pigments may have arisen in vegetative tissues in response to increased ultraviolet (UV) light and drought stress and were then subsequently co-opted by flowers to attract pollinators. In many cases, these pigments still maintain their original stress-related functions while also attracting pollinators.

The novelty of the results reported by Hanley *et al.* relies on the repeated evolutionary transitions in both plant defense and pollinator shifts. Here, convergence provides the evolutionary

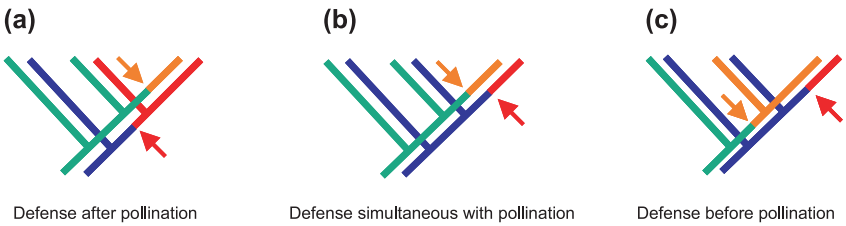

Fig. 1 The order of evolution in pollinator shifts and plant defense can be tested phylogenetically. Transitions between insect pollination (blue) and bird pollination (red) are indicated with red arrows. Transitions between low chemically defended lineages and high chemically defended lineages are indicated with an orange arrow. Pollinator shifts can occur before (a), simultaneous with (b), or after (c) the evolution of high cyanide levels. Hanley *et al*. describe a case of plant defense pre-adapting lineages to pollinator shifts (as in c).

replication necessary to test rigorously for correlations and to detect pre-adaptations (Fig. 1). A previous example of pre-adaptation for pollinator shifts mediated through plant defense traits has been described in *Dalechampia* (Armbruster, 1997), where plant resins, originally functioning in floral defense, were co-opted to provide rewards for resin-collecting bees. After the resins were co-opted for reward purposes, the flowers were presumably exposed to florivory, leading to the subsequent evolution of four secondary floral defense traits. The re-evolution of plant defense in *Dalechampia* emphasizes the constant selective pressures imposed by herbivores. In *Dalechampia*, and now in *Hakea*, we see floral traits associated with plant defense evolve before pollinator-driven floral evolution, questioning the primacy of pollinators in floral evolution.

Nonpollinator agents of selection on floral traits

Many recent studies on individual species have identified additional selective forces on flowers besides pollinators (Strauss & Whittall, 2006). In addition to influencing floral morphology, nonpollinator agents are also believed to play a role in mating systems and gender evolution (Ashman, 2002), reproductive interactions among plant species (Carlson *et al*., 2008) and community structure (see McCall & Irwin, 2006). Studies of nonpollinator agents frequently rely on species with polymorphisms in some floral trait(s) that may reflect the antagonistic selective forces of pollinators and other agents of selection. For example, the persistence of four color morphs in *Raphanus sativus* has been attributed to the opposing selective pressures of pollinators and herbivores. The yellow morphs (anthocyanin-less) are preferred by pollinators, yet are also the most palatable when presented to a diversity of herbivores. The increased herbivory in anthocyanin-less morphs may be attributed to their impaired inducibility of floral defense chemicals when compared with anthocyanin-producing morphs. The antagonistic roles of pollinators and herbivores are not unique to *Raphanus*; there is now a litany of examples suggesting that nonpollinator agents of selection affect various floral traits (reviewed in Strauss & Whittall, 2006).

In addition to biotic factors, such as herbivory, abiotic factors (including drought, UV light and heat-stress) have also been shown to affect floral-trait evolution, particularly flower color. Historically, when flower color polymorphisms could not be attributed to pollinator preferences, they were often considered to be evolutionarily neutral, as in the highly influential work on genetic drift in *Linanthus parryae* (Wright, 1943). Recent reconsideration of nonpollinator agents of selection in this case has revealed a negative correlation between the frequency of purple-flowered morphs and annual precipitation (Schemske & Bierzychudek, 2001). In a complementary manipulative study of five species from the British flora with flower color polymorphisms, pigmented morphs had higher fitness than nonpigmented morphs under artificially induced drought stress (Warren & Mackenzie, 2001). In many cases, during the co-option of vegetative anthocyanins by flowers, expression in these two tissues has not been completely decoupled and thus selection on vegetative tissues could influence flower color, and vice versa, through pleiotropy. The discovery of many biotic and abiotic nonpollinator agents of selection on flowers encourages a broader and more integrative ecological and genetic perspective on floral evolution – one that raises many new and exciting questions.

Future directions

The relative importance of pollinators and nonpollinator agents of selection in the evolution of floral traits is expected to vary according to ecological context, but the ecological parameters that weight the tug-of-war between these forces remain undefined. To determine the relative intensities of selection by pollinators and nonpollinator agents, and to identify the floral traits they target (i.e. flower color, flower size, reward, etc.), we could look to environments near the physiological limits of angiosperms. Here, abiotic selective pressures are predicted to be paramount. Arctic and alpine habitats are two such environments characterized by extreme environmental conditions in which the interactions of plants with insect herbivores and pollinators have been considered relatively rare (Mosquin, 1966; Tieszen, 1978). At these extremes, we expect to see floral traits swayed towards the primacy of nonpollinator agents of selection. Along transects into these extreme regions, we expect to see a cline revealing the intensity of selection by

Table 1 The percentage of species with anthocyanin-pigmented flowers in three genera divided into temperate, boreal and arctic regions

	Percentage of species with pigmented flowers		
	Temperate	Boreal	Arctic
Astragalus (*n* = 106)	42%	50%	75%
Oxytropis (*n* = 71)	57%	65%	67%
Cardamine (*n* = 18)	28%	40%	56%

An increasing percentage of species with pigmented flowers as latitude increases is consistent with the role of anthocyanins in increasingly stressful environments.

nonpollinator agents. In a preliminary comparative study of flower color frequencies in three genera spanning the arctic, boreal and temperate zones, we found that the frequency of species with anthocyanin-pigmented flowers increased with increasing latitude (Table 1), consistent with the primary function of anthocyanins in stressful environments. More detailed investigation into the phylogenetic relationships within these lineages, accompanied by manipulative experiments, would reveal the evolutionary history of this apparent pattern and its underlying causes.

An alternative approach to deciphering the roles of pollinators, herbivores and abiotic agents of selection on floral-trait evolution is to remove pollinators and herbivores as selective agents. This can be achieved using exclusion and hand-pollination experiments in taxa with variation in appropriate floral traits, then assessing the fitness effects and evolutionary consequences. This approach is expected to take numerous generations to detect changes in the frequencies of floral traits (c.f. Strauss *et al.*, 2008). Alternatively, we can utilize lineages that no longer rely on biotic pollinators, such as wind-pollinated taxa. When we see homologous floral traits maintained in these taxa that are typically attributed to pollinator attraction, reward or efficiency, we may turn our attention to alternative selective forces. For example, in wind-pollinated *Plantago lanceolata*, floral anthocyanins show increased inducibility at higher latitudes – consistent with the needs of these plants to maintain high metabolic rates to complete seed maturation in an abbreviated growing season (Stiles *et al.*, 2007).

Molecular investigations into the genetic basis of floral traits could also contribute to a broader understanding of the multifaceted evolutionary history of flowers. Previous studies have shown correlations between vegetative traits and floral traits (such as flower color) that may be attributed to the molecular constraints of gene expression across plant tissues (Armbruster, 2002). It is as yet unclear how often flower color shifts correlate with changes in vegetative anthocyanins or their flavonoid precursors. Another poorly understood aspect of floral-trait evolution is their ability to respond to selection, while other floral or vegetative traits are facing different selective regimes. We can now better quantify the evolvability of floral characteristics and compare pollinator-related and herbivore-related traits to assess which are more evolutionarily malleable (see Hansen *et al.*, 2003).

In general, further genetic investigations on individual species and their ecological contexts will reveal how the antagonistic and mutualistic forces of pollinators and nonpollinator agents, such as herbivores, shape floral evolution in a continuous and interactive manner. When combined with broader phylogenetic perspectives of the order and contingencies of floral-trait evolution, we envision many additional studies to complement the findings of Hanley *et al.*. We expect studies such as these will address the interplay of edibility, defense, abiotic stress and pollination services in generating and molding floral diversity.

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Key words: angiosperms, anthocyanins, diversification, floral evolution, plant defense, pollinator shifts, pre-adaptation.

Letters

Is it better to give than to receive? A stable isotope perspective on orchid–fungal carbon transport in the green orchid species *Goodyera repens* and *Goodyera oblongifolia*

In the field of orchid research, species within the tribe Cranichideae have taken center stage as a result of the recent findings of Cameron *et al.* (2006, 2008), which demonstrated carbon transport from adult *Goodyera repens* (L.) R. Br. orchids to their mycorrhizal fungus *Ceratobasidium cornigerum* (Bourdot) D. P. Rogers. The dependence of orchids in their early stages of development on fungi is a long-recognized trait of the family (Bernard, 1909; Dearnaley, 2007). However, there has been much controversy over the potential for carbon 'repayment' to the fungi once the orchid has formed leaves and is capable of assimilating its own carbohydrates through photosynthesis (Alexander & Hadley, 1985; McCormick *et al.*, 2006; Smith & Read, 2008).

Using ^{14}C-labeled carbon fed either to the mycelia of the orchid's fungal symbiont or to the plant as $^{14}CO_2$, Cameron *et al.* (2008) were able to quantify the carbon transport between the orchid and fungus over an 8-d period. Their findings were that the net transfer of carbon from *G. repens* to *C. cornigerum* was over five times greater than that of carbon transported from the fungus to the plant. While this extremely well-executed study provides the 'first full bidirectional C budget for any mycorrhizal association' (Cameron *et al.*, 2008), there are some limitations of their model and methods that must be taken into account. As mentioned in their recent article and the commentary by Johnson (2008), the carbon allocation to fungal biomass within the orchid's roots cannot be separated from that to the roots alone; nor can carbon respiration from the plant versus that from the fungus. Furthermore, the use of radiocarbon labeling gives measurements of carbon flow within a system for only a relatively short period of time. Also, as many of these labeling experiments are carried out in the laboratory, it is difficult to relate results to any field setting. A complementary method that has been applied to examine carbon and nitrogen gains from fungi by partially and fully myco-heterotrophic plants associated with ectomycorrhizal (ECM) and litter- or wood-decaying saprotrophic (SAP) fungi is the use of naturally occurring stable isotopes of carbon and nitrogen (^{13}C:^{12}C and ^{15}N:^{14}N) (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Ogura-Tsujita *et al.*, 2009). Measured isotope abundances are denoted as δ values and are calculated according to the equation: $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. In contrast to radiocarbon labeling, the analysis of the bulk carbon isotope values of field-collected plants gives an integrated view of carbon assimilation throughout the period during which the tissue was synthesized (Dawson *et al.*, 2002).

While there is a subset of orchid species that remain myco-heterotrophic for their entire life cycle and lack the ability to photosynthesize (Leake, 1994), it was previously believed that green species are completely released from their dependence on heterotrophic carbon gain once leaves are formed (Alexander & Hadley, 1985). However, recent analysis of the carbon and nitrogen isotope signatures of some green orchids has revealed

Table 1 Sampling locations in the USA (CA, OR) and Austria (Vorarlberg) including *Goodyera* and reference species collected (*n*, number of replicates), and mean (± 1 SD) $\delta^{15}N$ and $\delta^{13}C$ values (‰) in leaves of *Goodyera* and reference species

Location	Species (*n*)	$\delta^{15}N \pm 1$ SD	$\delta^{13}C \pm 1$ SD
El Dorado National Forest, CA	*Goodyera oblongifolia* (4)	–2.4 ± 0.8	–32.3 ± 0.8
38°54′01.70″N 120°34′26.77″W	*Abies concolor* (5)	–3.4 ± 0.9	–31.0 ± 0.3
El Dorado National Forest, CA	*G. oblongifolia* (5)	–3.2 ± 0.8	–33.3 ± 1.2
38°54′3.47″N 120°34′28.40″W	*A. concolor* (5)	–4.0 ± 0.7	–30.6 ± 0.7
	Ribes roezlii (5)	–4.3 ± 1.2	–31.4 ± 0.6
Plumas National Forest, CA	*G. oblongifolia* (5)	–2.0 ± 1.1	–33.2 ± 0.7
40°03′36.02″N 120°51′32.99″W	*A. concolor* (5)	–3.8 ± 1.1	–30.4 ± 1.0
Willamette National Forest, OR	*G. oblongifolia* (1)	–1.6	–33.4
44°18′36.00″N 122°00′36.02″W	*Tsuga heterophylla* (5)	–2.2 ± 0.8	–31.9 ± 1.9
Marultal, Vorarlberg	*G. repens* (5)	–6.2 ± 1.1	–36.6 ± 1.9
47°11′44″N 9°53′57″E	*Knautia sylvatica* (5)	–8.9 ± 0.8	–32.1 ± 0.6
	Mercurialis perennis (5)	–7.7 ± 0.6	–30.3 ± 0.7
	Vaccinium vitis-idaea (5)	–9.7 ± 0.9	–30.5 ± 0.5

that many of these putative autotrophic orchids that associate with ECM fungi actually still partially rely on these fungi to meet their carbon demands. These orchid species have been referred to as mixotrophs or partial myco-heterotrophs. Unlike obligate myco-heterotrophic orchids, which have $\delta^{13}C$ signatures most similar to those of their fungal symbionts, mixotrophic orchids tend to have $\delta^{13}C$ signatures intermediate between those of surrounding autotrophic and myco-heterotrophic plants (Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007).

Interestingly, an additional category of orchids that are depleted in ^{13}C compared with surrounding autotrophic plants is emerging from recent stable isotope analysis of species in the closely related tribes Orchideae and Cranichideae (H. T. Liebel *et al.*, unpublished), the latter containing the genus *Goodyera* (data herein). We collected leaf samples of *Goodyera oblongifolia* Raf. from four sites in northern California and southern Oregon, USA, and *G. repens* from a single site in the Austrian Alps (Table 1). The site in the Alps was an open rocky outcrop habitat, while all samples collected in the USA were from the deeply shaded understories of mixed conifer forests. Altogether, leaves of 15 *G. oblongifolia* and five *G. repens* individuals were collected. In addition, at each sampling site a minimum of five autotrophic individuals from at least one species were collected for a total of 40 individuals of six species (Table 1). These collections were used as reference plants representative of the autotrophic understory.

The collected plant samples were then analyzed for carbon and nitrogen stable isotope abundances via elemental analyzer/continuous flow isotope ratio mass spectrometry at either the Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), Germany or the Center for Stable Isotope Biogeochemistry at University of California Berkeley, as described in Zimmer *et al.* (2007). Once δ values were obtained for all samples from the USA (Table 1), the $\delta^{15}N$ and $\delta^{13}C$ values of all reference plants were tested for inter-site variation with a one-way ANOVA and Tukey's Honestly Significant Differences (HSD). Because of significant differences at an α value of 0.05 in the $\delta^{15}N$ values of the reference plants between two sites in California ($P = 0.007$), the δ values from the USA could not be pooled to make comparisons across sites between *Goodyera* samples and their respective references. To make these comparisons, δ values for both elements and all samples collected in the USA and at the single Austrian site (for consistency) were converted into site-independent enrichment factors (ε) and pooled based on species identity and location (USA or Austria). The calculation of ε factors systematically eliminates the majority of the influence of spatial variation on δ values resulting from site-specific differences in carbon and nitrogen isotope abundances, thus allowing comparisons of these values across sites (Emmett *et al.*, 1998; Gebauer & Taylor, 1999; Preiss & Gebauer, 2008). First, for each site the $\delta^{13}C$ and $\delta^{15}N$ values of reference plants were averaged. Then, on a per site basis, these averages were subtracted from the $\delta^{13}C$ and $\delta^{15}N$ values of the *Goodyera* samples and reference plants to create site-independent enrichment factors ($\varepsilon = \delta x_S - \delta x_R$) for each sample (where $\delta x_S = \delta^{13}C$ or $\delta^{15}N$ of individual samples per site and δx_R = mean $\delta^{13}C$ or $\delta^{15}N$ of all reference plants per site). The resulting mean of both ^{13}C and ^{15}N ε factors of the autotrophic reference plants was equal to 0‰. However, the enrichment factors of individual reference plants clustered at *c*. 0‰, reflecting the small inter- and intraspecific variations in their isotope signatures, which were not significantly different between sites. The two *Goodyera* species' ε factors species separated as distinct groups for both elements based on the differences of their δ values from the mean of their respective references (Fig. 1). The variance around the mean $\delta^{13}C$ or $\delta^{15}N$ values of reference plants used to calculate $\varepsilon^{15}N$ and $\varepsilon^{13}C$ was retained by calculating ε factors for not only both *Goodyera* species, but also reference

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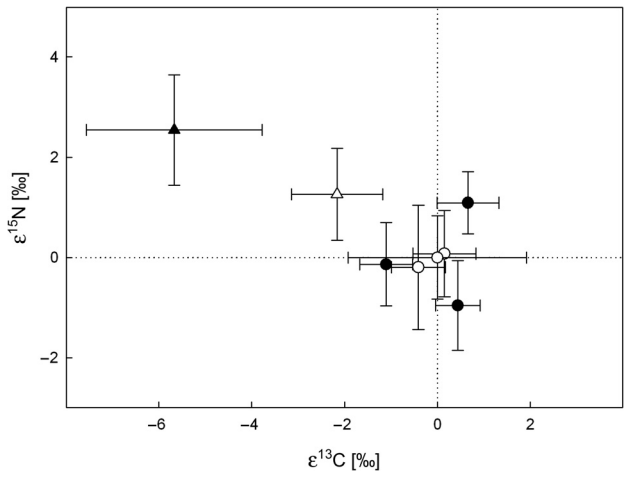

Fig. 1 Mean enrichment factors (ε) of ^{13}C and ^{15}N from the leaves of *Goodyera oblongifolia* (open triangle), *Goodyera repens* (closed triangle) and each species of autotrophic reference plants collected in the USA (open circles) and in Austria (closed circles). Error bars indicate 1 SD for each *Goodyera* species and their respective reference plants.

plants on a site by site basis. Statistical comparisons between the individual enrichment factors of individual *G. repens* and *G. oblongifolia* plants and their respective autotrophic references from either Austria or western USA were made using Mann–Whitney *U*-tests. Both *G. repens* ($P = 0.002$) and *G. oblongifolia* ($P = 0.008$) were significantly enriched in ^{15}N compared with surrounding autotrophic plants (Fig. 1). By contrast, both *Goodyera* species were significantly depleted in ^{13}C in comparison to their references ($P < 0.001$; Fig. 1). *Goodyera repens* plants from the open sunny habitat in the Alps were considerably more depleted in ^{13}C compared with *G. oblongifolia* from deeply shaded forests.

Although the sample size of *G. oblongifolia* and *G. repens* individuals collected in this study were relatively small, the stable isotope evidence presented here shows that these orchids do not exhibit any trends toward full or partial myco-heterotrophy. In fact, these orchids' consistent depletion in ^{13}C compared with surrounding autotrophic plants reveals a distinct nutritional strategy. The physiological mechanism leading to this depletion remains unknown, but may be related to the transfer of ^{13}C-enriched carbon compounds from these orchids to their associated fungi (*sensu* Gleixner *et al.*, 1993). This would fit well with Cameron *et al.*'s (2006, 2008) findings of carbon transfer from orchid to fungus, as well as with isotope food-chain models where the source of a nutrient is left depleted in the heavy isotope compared with the sink (Fry, 2006). What is unclear is why *Goodyera* species would be significantly more depleted in ^{13}C than surrounding autotrophic mycorrhizal plants that are transferring substantial amounts of carbon to their fungal symbionts (Smith & Read, 2008). Habitat may also play a key role in determining the ^{13}C enrichment factors of *Goodyera* species. For instance, there exists some evidence that green orchids capable of partial myco-heterotrophy increase their dependence on fungal assimilated carbon when in deeply shaded habitats, leading their leaf $\delta^{13}C$ values to become more enriched than those of surrounding autotrophic plants (Bidartondo *et al.*, 2004; McCormick *et al.*, 2004; Zimmer *et al.*, 2007). If *G. oblongifolia* individuals from our forested sites were at an earlier stage of seedling development more dependent on heterotrophic carbon gain than *G. repens* from open sites, then this could explain why the former is less depleted in ^{13}C than the latter. The significant enrichment in ^{15}N (a hallmark of all myco-heterotrophic orchids studied to date) found in both *Goodyera* species supports this theory, and Cameron *et al.*'s (2008) statement that these orchids are more parasitic upon their fungal symbionts than other mycorrhizal plants and therefore may govern the amount of nutrient exchange to the fungus. This idea of 'orchid control' over its mycorrhizal associations is further exemplified by the unique morphology of orchid mycorrhizas, where fungi that are known to be saprotrophic or ectomycorrhizal when independent of orchids form intracellular coils when in association with orchids (Rasmussen, 2002).

Cameron *et al.*'s (2006, 2008) work provides the first example, in *G. repens*, of an orchid species that, upon becoming photosynthetically active, can transfer carbon back to its mycorrhizal fungus. Unlike other green orchids studied to date, species within the tribes Orchideae and Cranichideae, including *G. repens* and *G. oblongifolia*, are the first species found to be depleted in ^{13}C compared with surrounding autotrophic plants (H. T. Liebel *et al.*, unpublished and data herein). In summary, based on carbon stable isotope abundances and the identity of their mycorrhizal associates, it is now clear that terrestrial orchids can utilize at least four nutritional strategies: autotrophy, where green orchids have carbon isotope signatures indistinguishable from those of surrounding autotrophs and mainly associate with *Rhizoctonia* species (a polyphyletic group of fungi); partial myco-heterotrophy, where green orchids have carbon isotope signatures intermediate between those of autotrophs and myco-heterotrophs and associate with ECM fungi; obligate myco-heterotrophy, where orchids have lost the ability to photosynthesize, are specialized on either ECM or SAP fungi, and are enriched in ^{13}C, similar to their host fungi; and an additional strategy found in green orchids from the tribes Orchideae and Cranichideae, which mainly associate with ceratobasidioid and tulasnelloid fungi and are depleted in ^{13}C compared with surrounding autotrophs (Fig. 1 and H. T. Liebel *et al.*, unpublished). The variability of the ecology and physiology of orchids is not surprising, as orchids are the largest plant family, whose evolutionary history potentially stretches back to the late Cretaceous (Ramírez *et al.*, 2007). While there is still much to discover about the intriguing Orchidaceae, combining the use of naturally abundant isotopes and radioactive tracers along with molecular methods, especially those that allow comparisons at the genotype level (Johnson, 2008), will continue to help us understand the links between the evolutionary history of orchids, their physiology and interactions with fungi.

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Key words: ^{13}C, ^{15}N, *Goodyera*, mycorrhiza, orchids, stable isotopes.

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Meetings

Individuals, populations, communities and function: the growing field of ectomycorrhizal ecology

21st New Phytologist Symposium: The ecology of ectomycorrhizal fungi, Montpellier, France, December 2008.

The 21st New Phytologist symposium entitled 'The ecology of ectomycorrhizal fungi' attracted over 100 participants to Montpellier, France, for a two-day meeting in early December. Marc-André Selosse (Université Montpellier, France) and Ian Alexander (University of Aberdeen, UK) organized the talks around the classic ecological hierarchy of individuals, populations and communities. The meeting also struck a balance between trying to apply broad ecological theory to ectomycorrhizal fungi and investigating the unique aspects of the organisms and the interactions involved.

'If this pattern holds, it has major implications for the larger field of ecology because it would indicate that ectomycorrhizal fungi represent a rare exception to the negative correlation between diversity and latitude.'

Mark Fricker's (Oxford University, UK) innovative work on fungal networks provided an excellent example of the type of organism-specific research that is crucial to the field. His time-lapsed, false-color videos of the movement of radioactively labeled carbon-containing and nitrogen-containing compounds captivated the audience. The finding of oscillatory pulses in fungal transport and their synchrony when networks fuse was particularly striking and required the development of sophisticated image analyses to be demonstrated (Fricker *et al.*, 2007). All of his work has thus far been conducted using saprobic fungi, but it is likely to be highly applicable to ectomycorrhizal systems.

Population ecology

In the symposium's introduction, Ian Alexander (University of Aberdeen, UK) noted that population ecology has not been well represented in the field of ectomycorrhizal ecology but the organizers arranged for three talks to review recently published work. Andre Rubini's (Plant Genetics Institute, Perugia, Italy) presentation on the economically important truffles, *Tuber magnatum* and *Tuber melanospora*, provided an excellent example of how population genetics can lead to autecological knowledge. Early studies on *T. magnatum* had suggested that the species was characterized by selfing because single sporocarps always appeared to be homozygous for polymorphic markers (Bertault *et al.*, 2001). However, Rubini showed that this result was an artifact of the DNA-extraction method that preferentially extracted the haploid parental tissue and failed to extract the recombinant thick-walled ascospores. After adapting more vigorous extractions methods, it was found that that populations were outcrossing, diverse and geographically structured (Paolocci *et al.*, 2006, Riccioni *et al.*, 2008). Similarly, Annette Kretzer (SUNY-ESF, NY, USA) used microsatellite markers to explore the autecology of two false-truffles in the genus *Rhizopogon* and showed that they differed from each other in the size of their vegetative individuals (i.e. genets). This result was extended in a poster by Beiler *et al.* (University of British Columbia, Canada) who simultaneously genotyped roots of individual trees and showed that individuals of both *Rhizopogon* species associated with multiple trees. Kretzer also applied refined analytical methods to try to discern parent/offspring relationships among the *Rhizopogon* genets, but had limited success as a result of the resolution provided by the markers available (Kretzer *et al.*, 2005). However, the posters by Labbé *et al.* (INRA/Nancy Université, France) and Vincenot *et al.* (Université Montpellier, France) on *Laccaria* species showed that as the genomic era unfolds, the days of limited resolution may be over. Making use of the entire genome of *Laccaria bicolor*, they developed a large number of simple sequence repeat markers in both *L. bicolor* and *Laccaria amethystina* and applied these to Eurasian collections of *L. amethystina.*

It became clear from discussions that few generalizations could be made about fungal populations. Some fungi, such as *Tuber* and *Rhizopogon*, show geographic structure on a fairly fine scale, and others, such as *Laccaria*, have large well-mixed

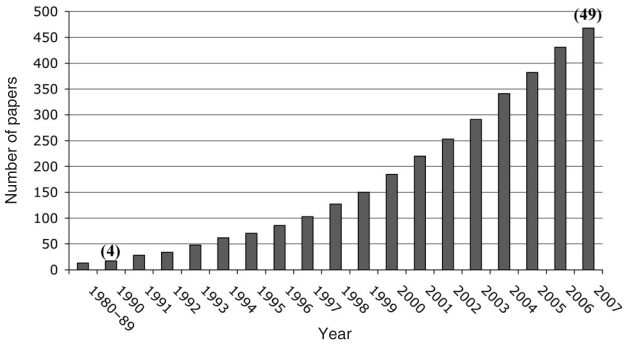

Fig. 1 Accumulation of publications on ectomycorrhizal community ecology.The results are from a Biosis search for Ectomycorr* and Commun* and Ecol*. Almost 500 papers have now been published on the subject. Forty-nine of these were in the last completed year (2007), compared with four in 1990.

populations across Europe. Is this caused by differences in the mode of spore dispersal (i.e. animals vs wind)? Or will this pattern break down as more species are sampled? However, the presence of cryptic species (i.e. species that look nearly identical but are genetically isolated) appears to be widespread in fungi. Kretzer's two *Rhizopogon* species were an example of this, as they were originally thought to be a single species. Many additional examples were provided in Greg Douhan's (University of California, Riverside, USA) and Andy Taylor's (Macaulay Institute and University of Aberdeen, UK) presentations. Taylor's talk was especially interesting because he showed many examples of cryptic species in the genus *Xerocomus.* As if to underline how poorly known these common fungi are, one of his undescribed species was found right in Silwood Park (Taylor *et al.*, 2007), but more interestingly, most of these species had distinct geographic distributions and host preferences. Using molecular clock estimates, Taylor postulated that these geographic distributions fit with distinct geological events, such as the final split up of Laurasia (60 million years ago) or the more recent Beringia land bridge (14 000 years ago), and he thus tied their autecology back to more general biogeographic patterns.

Community ecology

Ectomycorrhizal community ecology has received more attention than any other subdiscipline within the field. Publications on the topic have exhibited a 12-fold increase over the last 20 yr (Fig. 1). Progress in this area is largely a result of the increased ability to identify and quantify ectomycorrhizal fungi below ground using molecular methods (Horton & Bruns, 2001). While the patterning of ectomycorrhizal communities has been documented in increasing detail, this subdiscipline has been lacking a global perspective (Dickie & Moyersoen, 2008) and a mechanistic or theoretic understanding of what drives community assembly and structure.

Host-plant richness has long been thought to influence ectomycorrhizal community structure and diversity significantly, and a meta-analysis by Dickie (2007), supported this idea by identifying a strong, positive correlation between the richness of host plants and ectomycorrhizal fungi. However, few studies explicitly examined if the phylogenetic diversity of host plants correlates with ectomycorrhizal community structure and diversity. Kazuhide Nara (University of Tokyo, Japan) presented recent work showing that ectomycorrhizal communities on phylogenetically similar hosts were significantly more similar to each other than those on less closely related hosts (Ishida *et al.*, 2007), and found that this correlation was caused mostly by host preference rather than absolute specificity. To determine if similar richness relationships were present in tropical forests, Dr Nara examined the ectomycorrhizal community structure and diversity in an Indonesian dipterocarp-dominated forest with high host-plant diversity. He found that ectomycorrhizal diversity was much lower than predicted from temperate studies and that his results were similar to those of the handful of other studies carried out on tropical ectomycorrhizal communities (Tedersoo *et al.*, 2007). If this pattern holds, it has major implications for the larger field of ecology because it would indicate that ectomycorrhizal fungi represent a rare exception to the negative correlation between diversity and latitude. Confirming this result and understanding why ectomycorrhizal diversity patterns may differ from other organisms are clearly ripe areas for future research.

Community ecology is certainly a well-developed field in other systems, and dispersal, environmental conditions and biotic interactions have all been shown to play important roles in the assembly and dynamics of plant and animal communities. Peter Kennedy (Lewis and Clark College, USA) examined the roles of these factors in ectomycorrhizal communities and suggested that their relative importance in community assembly is both spatially and temporally scale-dependent. He also presented work showing that the assembly of ectomycorrhizal communities can be strongly influenced by the order and spatial patterning of species arrival. Another example of the growing confluence between ectomycorrhizal and general community ecology was demonstrated by Sara Branco (University of Chicago, USA), whose poster won top prize at the symposium. Ms Branco used an ecophylogenetic approach (Webb, 2000) to examine the community dynamics of ectomycorrhizal fungi present in serpentine soils. She found strong phylogenetic clustering in comparisons between serpentine and nonserpentine communities, but strikingly different patterns of clustering at the species and genus levels. From this part of the symposium, it was clear that the widespread adoption of molecular techniques has allowed ectomycorrhizal ecologists to describe many aspects of communities, but a combination of greater experimentation (particularly in the field) and theory-based research questions are now needed to move the subdiscipline towards greater synthesis and predictive ability.

Given the high diversity found in most ectomycorrhizal fungal communities, examining functional differences among species is important to integrate the parts of the aforementioned ecological hierarchy (Koide *et al.*, 2007). Jean Garbaye (INRA, Nancy, France) presented recent work linking taxonomic diversity with the diversity of enzymatic activity associated with individual ectomycorrhizal root tips. The method assays individual excised root tips for multiple enzymatic activities with a microtiter dish format (Courty *et al.*, 2005). At the community level, Garbaye demonstrated that rare ectomycorrhizal species often contributed disproportionately to total enzymatic activity, indicating their potential importance for ecosystem functioning. He also showed that enzyme activity varied considerably within species depending on environmental conditions, soil depth and time of year. A major attraction of this assay is that it has a very high throughput; however, the removal of extraradical hyphae during tip preparation creates an artifact that complicates comparisons among species. An alternative way to assay enzymatic activity under field conditions was presented by Brooks *et al.* (University of British Columbia, Canada) in a poster. This technique involved taking an imprint of the enzymes from a soil profile onto a membrane, assaying the activity of selected enzymes (in this case phosphatase) and correlating it with the ectomycorrhizal fungi or other organisms present at that particular point in the soil. While this method allows one to assay the contributions of extramatrical hyphae along with other soil organisms, it is not well suited to the same high-throughput analyses that the excised tip method offers. Thus, like other methods in this field, these approaches provide useful, but limited, views into this complex symbiosis.

The small meeting format and the limited number of talks provided many opportunities for lively discussions. These were enhanced by the frequent comments from Sir David Read (University of Sheffield, UK), whose voluminous work on mycorrhiza laid the foundation for much of the current research (Alexander, 2007). Overall, the continuity between past and present, methods and theory, and opinions and data made this meeting a productive step towards the future in this vibrant field. The program and the abstracts are available on the web for those who were unable to attend (http://www.newphytologist.org/mycorrhizal/default.htm).

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Key words: community, ecology, ectomycorrhiza, fungi, genetics, population.

Mycorrhizas in tropical forests: a neglected research imperative

Mycorrhizas in Tropical Forests, a workshop held at Universidad Técnica Particular de Loja (UTPL), Loja, Ecuador September, 2008

Mycorrhizal research in tropical forest has a long history. Janse's (1896) paper of 'Les endophytes radicaux de quelques

plantes javanaises' was published a mere 11 years after Frank (1885) first coined the term 'mycorhiza'. It contains not only some of the first accurate depictions of arbuscular, orchid and ericoid mycorrhizas, but also detailed descriptions of the mycorrhizas of both orchidaceous and nonorchidaceous myco-heterotrophic plants. Even in Janse's time, the presence of many different mycorrhizal interactions on co-existing, but phylogenetically distant, tropical forest plants was apparent. Inexplicably, given the ecological and economic importance of tropical forest, and the probable role of mycorrhizas in maintaining their biodiversity and productivity (Alexander & Lee, 2005), the mycorrhizas of tropical forest plants did not attract much attention in the next 100 years. Indeed, even in this century, only 170 of the 5600 papers published on mycorrhizas since 2000 refer explicitly to tropical forest (Fig. 1). However, things may be about to change, as the attention of mycorrhizal researchers turns belatedly to the threats to biodiversity in tropical forests, their importance in understanding the evolution and biogeography of mycorrhizal fungi and their pivotal role in the earth's carbon cycle and climate system (Gilbert & Strong, 2007). The workshop on 'Mycorrhizas in Tropical Forests' held in Loja, Ecuador, September 2008, was therefore particularly timely. Twenty-six participants from 12 countries attended the workshop, which included presentations, poster sessions, field excursions and many free-ranging discussions. Abstracts of all the talks and posters, and details of the participants, can be found on the website (http://www.mycorrhiza-research.de/Workshop/01Welcome.html).

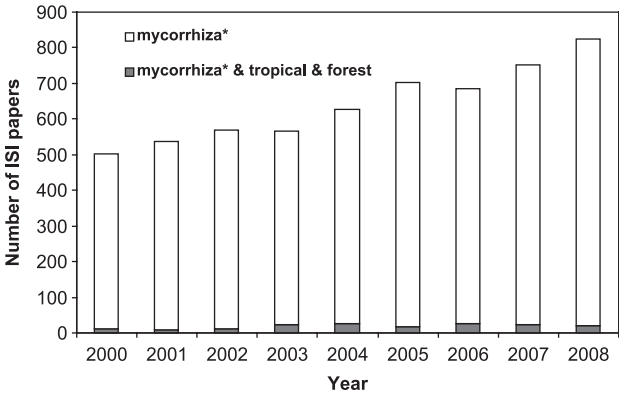

Fig. 1 The number of ISI journal papers from 2000 to 2008 dealing with mycorrhizas in tropical forest (search string: mycorrhiza* & tropical & forest), compared with the total mycorrhizal literature (search string: mycorrhiza*).

'... we know that mycorrhizal fungal communities in undisturbed tropical forest can be complex and species rich, we do not understand how important that complexity is to forest diversity, productivity and resilience.'

In his opening lecture, Ian Alexander (University of Aberdeen, UK) introduced four broad themes: the link between mycorrhizal fungal community composition and ecosystem processes; the biogeography of tropical mycorrhizas; the importance of fungal taxonomy; and the challenge of demonstrating the relevance of mycorrhizal fungal diversity to forest resilience and restoration. There followed much debate about the relative importance of biotic and edaphic factors in determining the composition of mycorrhizal fungal communities of tropical trees, and the significance of fungal community composition to the composition of tree regeneration. Scott Mangan (Smithsonian Tropical Research Institute, Panama) showed that spatial differences in arbuscular mycorrhizal fungal (AMF) communities affected the composition of tree seedling communities in Panama and that there were strong feedbacks between hosts and AMF fungi. Jean Weber (Institute of Tropical Forestry and Forest Products, University Putra Malaysia) used spatial statistics to show an inverse relationship between the density of ectomycorrhizal (ECM) *Shorea* seedlings and the density of parents in Malaysian dipterocarp forest. Amadou Bâ (Université des Antilles et de la Guyane, Guadeloupe) demonstrated how AMF increase the flooding tolerance of the wetland tree *Pterocarpus officinalis* Jacp. in Guadeloupe.

A number of participants highlighted how tropical forest studies can shed new light on the biogeography and evolution of mycorrhizal fungi (Thelephorales, Leho Tedersoo (University of Tartu, Estonia)); Sebacinales, Sabrina Setaro (Wake Forest University, NC, USA); Tulasnellales, Juan Pablo Suarez (Universidad Técnica Particular des Loja, Ecuador) and their hosts (Dipterocarpaceae, Bernard Moyersoen (Université de Liège, Belgium); Orchidaceae, Tupac Otero (Universidad Nacional de Colombia, Colombia)). There was wide-ranging discussion of the need for, value of and optimization of automated taxonomic methods to deal with the plethora of new, insufficiently identified, fungal sequences that are likely to result from increased environmental sampling in tropical forest (Markus Göker, Eberhard-Karls-University Tübingen, Germany), and the particular problems of, and ways to resolve, species recognition in AMF were highlighted (Arthur Schüssler, Ludwig-Maximilians-University Munich, Germany).

The enduring fascination of myco-heterotrophic plants was much in evidence. Stephan Imhof (University Marburg, Germany) showed how painstaking three-dimensional reconstruction reveals the complex ways in which nonphotosynthetic hosts manipulate development of AMF. Marc-André Selosse

(CEFE-CNRS, Université Montpellier II, France) hypothesized that higher availability of photosynthates for tropical ECM fungi leads to the reduced specificity he found in tropical mycoheterotrophic orchids; in the absence of suitable ECM fungi, saprobic fungi may even be sufficiently active under hot and wet conditions to act as carbon sources. Ingrid Kottke (Eberhard-Karls-University Tübingen, Germany) and her team demonstrated elegantly how careful morphological/anatomical studies on tropical plants coupled with molecular information can challenge our concepts of what constitutes an arbuscular, ecto- or ericoid mycorrhiza.

While tropical forests can still yield new and unexpected aspects of mycorrhizal biology, many questions fundamental to the very existence of the forest remain unanswered. For example, although we know that mycorrhizal fungal communities in undisturbed tropical forest can be complex and species rich, we do not understand how important that complexity is to forest diversity, productivity and resilience. Similarly, although we know that logging and forest conversion reduce fungal diversity, we have no hard evidence that reconstructing that diversity is important for restoration, or indeed how long it might take for the original community to reform. It was encouraging therefore that several participants addressed the potential for the practical application of mycorrhizal research in a range of locations (María Díez (Universidad Nacional de Colombia, Colombia) in Colombia; Tesfaye Wubet (Helmholtz Centre for Environmental Research, Halle, Germany) in Ethiopia; Ingeborg Haug (University Tübingen, Germany) in Ecuador; and Laura Aldrich-Wolfe (North Dakota State University, USA) in Costa Rica).

Loja proved to be an ideal location for the workshop, both for its ready access to the mega-diverse montane tropical rain forest and páramo of Southern Ecuador, and the example it provided of the benefits of international collaboration, as shown by the wealth of information emerging from this ecosystem as a result of the research programme on mycorrhizas in tropical forest established by Ingrid Kottke (Eberhard-Karls-University Tübingen, Germany) and Juan Pablo Suarez (Universidad Técnica Particular des Loja, Loja, Ecuador).

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Key words: biodiversity, fungal biogeography, fungal communities, mycorrhiza, tropical forest.