

## Commentary

# Remote control – cell and organ communication within plants

The fascinating thing about plants is their developmental flexibility, which to a remarkable extent is driven by the environment – both abiotic and biotic – that surrounds them. Plants react to these inputs by modifying both their physiological behaviour and their pattern, direction and intensity of growth. Physiological and developmental re-programming events inevitably require the co-ordinated action of cells and organs and, hence, regulatory information has to be transmitted between the interacting entities. Two papers in *New Phytologist* (Hanstein & Felle, pp. 591–602 in this issue; Thomas *et al.*, 2004) provide new approaches for investigating plant cell-to-cell and long-distance (systemic) signalling.

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*‘Simply imagine – we could modify the physiological status of one cell, monitor online what happens in the intercellular compartment, and detect what the neighbouring cells do’*

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### Bridging the single-cell to whole-tissue divide using nanoinfusion

Hanstein & Felle introduce the ‘nanoinfusion’ technique for studying the local response of a tissue. Three electrodes for measuring pH and voltage, and for infusion were positioned next to each other in three neighbouring substomatal cavities (Fig. 1). The suitability of the technique was demonstrated by measuring a significant pH change in response to elicitor application. The authors focus on the technical set-up, performing a range of control experiments and providing a clear picture in terms of practicality and reliability.

They have converted a relatively tricky technique into an operable system. By flooding the substomatal cavities, electrode positioning was made easier and less time consuming,

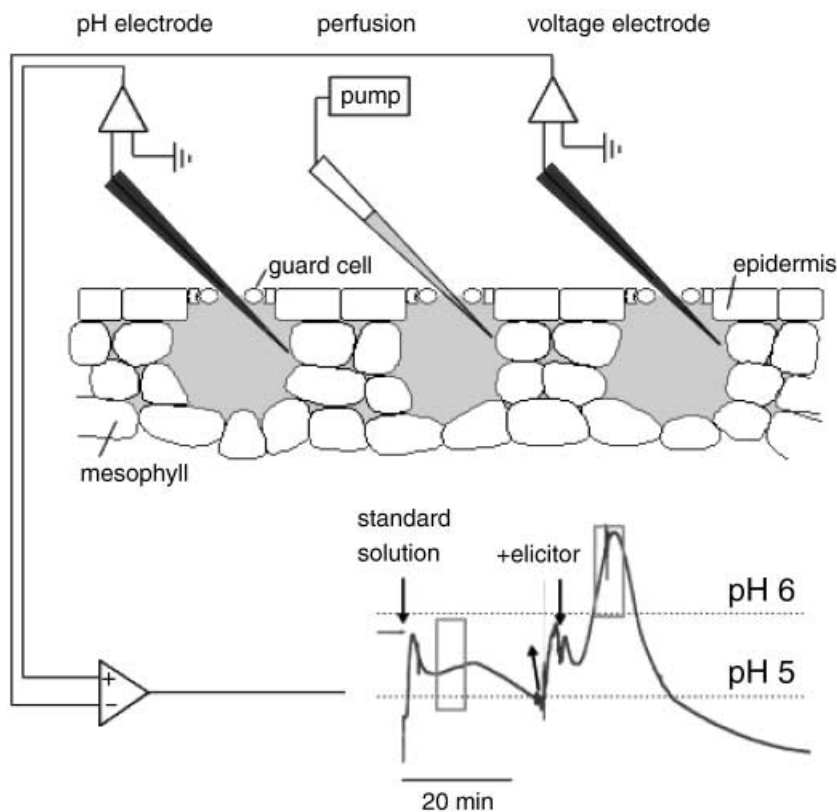
and application of the elicitor – which would hold true for other substances as well – was possible. Time resolution of pH measurement is fast. The duration of a measurement depends on the time of the plant’s response and, in the case of elicitor application, a characteristic pH change was registered within 20 min (Fig. 1). Conditions have been established and standardized for barley and could be brought forward to other species such as wine and poplar. The major advantage of nanoinfusion is that it is minimally invasive. All cells surrounding the stomatal cavity contribute to the response – the cells remain in their physiological state, unlike, for instance, in patch-clamp experiments, where it is possible to get detailed information about the response of a single cell, but at the expense of protoplast preparation. Nanoinfusion therefore fills a gap between single-cell and whole-tissue analysis.

Is nanoinfusion a useful screening system? First of all, the pH response of wild-type and a mutant barley plant differed (see Hanstein & Felle, Fig. 6), but with a throughput of three to four plants per day nanoinfusion can hardly be called a high-throughput screening system. However, it has potential as a screening system in a smaller context for the analysis of transgenic plants. A further interesting potentiality of nanoinfusion lies in its combination with pretreatment procedures in the development of resistance inducers for use in agriculture. In this context the technique would be used to monitor the status of the plant (resistant or nonresistant) attained via a systemic response.

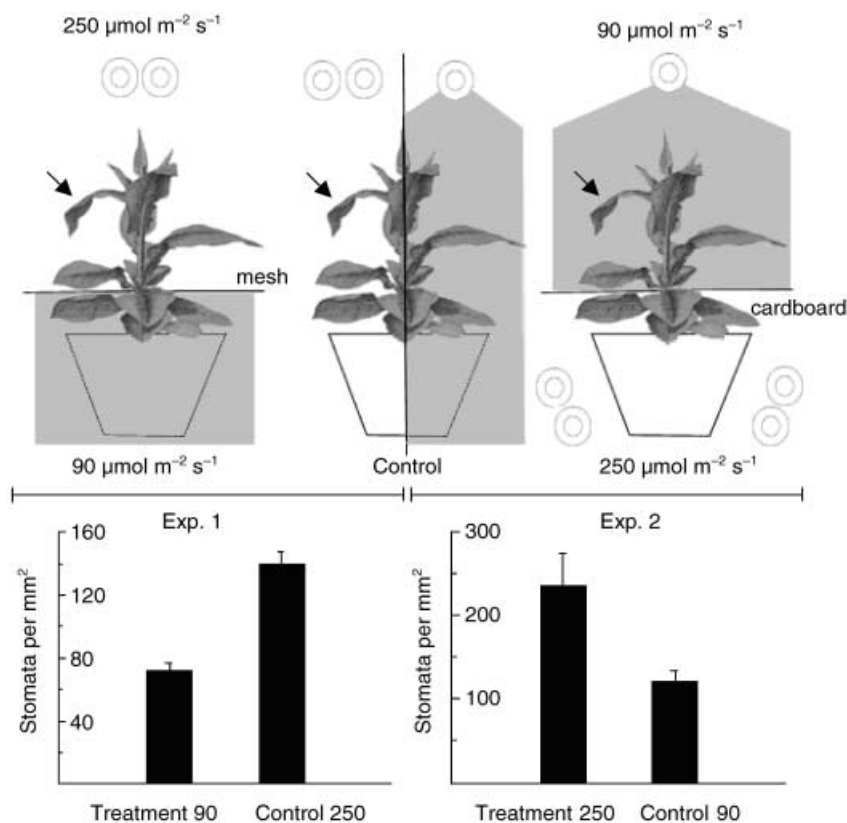
### Systematic signalling in leaf development

Thomas *et al.* (2004) moved away from classical leaf development models, where leaf development had been considered to be influenced by its local light environment without including possible ecological impacts, towards a more holistic view. The simple question was: does the irradiance that is sensed by mature leaves signal to newly emerging leaves to adjust their further development to the prevailing light conditions? This is an important issue from a physiological point of view – at the shoot apex the newly developing leaves are generally covered by other, more advanced leaves, and hence cannot exactly ‘measure’ the atmospheric conditions (light, CO<sub>2</sub> concentration and humidity) that surround the plant.

The authors combined the determination of several leaf parameters as a measure for development with a clever illumination set-up to monitor anatomical characteristics of developing tobacco leaves under different irradiance regimes (Fig. 2). In the newly developing leaves stomatal density, the stomatal index, epidermal cell shape, epidermal cell size and



**Fig. 1** Nanoinfusion. Sketch of the nanoinfusion set-up (modified after Hanstein & Felle, pp. 591–602 in this issue). Three micropipettes for pH measurement, perfusion and voltage measurement are positioned in adjacent stomatal cavities, which are flooded by applying pressure to the perfusion pipette. The pH is measured as the voltage difference of pH- and voltage electrodes, with the ground electrode placed in the solution in which the cut end of the leaf resides. A significant increase in pH occurred after application of an elicitor. The upward arrow indicates the necessary removal of the perfusion pipette before the second infusion.



**Fig. 2** Systemic irradiance signalling. Schematic presentation of the set-up used to prove that a systemic irradiance signal from mature leaves influences the anatomical characteristics of developing leaves (re-plotted from Thomas *et al.*, 2004). Initially, all plants were grown under high irradiance until a certain age, then mature leaves (experiment 1) or newly developing leaves (experiment 2) were shaded by using a mesh or by irradiating with two light intensities with cardboard as a shield. Control plants were grown under high or low irradiance, respectively. One of the newly developing leaves (indicated by the arrow) was analysed with respect to various developmental parameters. As an example, stomatal densities from abaxial surfaces of the treated and the control plants are presented.

developing leaf size were influenced by the irradiance signal administered to the mature leaves. To give an example, shading of the mature leaves alone resulted in lower stomatal density in the newly developing leaves, whereas illuminating the mature leaves with a higher irradiance had the opposite effect, leading to an elevated stomatal density in the new leaves that were shaded (Fig. 2). Therefore, the newly emerging leaves adapted to a light regime that could not directly be sensed by themselves, but rather was experienced by the distant mature leaves, indicating a systemic irradiance signalling system.

This is an extremely important new piece of information and raises interesting questions that reach into both the molecular and ecological directions. What is the makeup of the sensing system in mature leaves and what is the actual signal that is transmitted to new leaves? Is it a sugar, a hormone, an RNA or a protein, or another small molecule that remains to be discovered? Even if 'boring' sucrose is involved, the highly interesting question remains as to how this molecule could precisely direct leaf development and properly adjust it to the 'upcoming' environmental conditions. Does the information relay system require a change in gene activity in mature leaves? It certainly does so in the developing leaf, as sophisticated cell differentiation and tissue organisation have to be matched.

The current report extends a previous publication from the same group. Lake *et al.* (2001) demonstrated that, in *Arabidopsis*, atmospheric CO<sub>2</sub> concentration is sensed by mature leaves, determining the development of the stomata in younger leaves. Thus, the CO<sub>2</sub> response is another example of a systemic signalling process that regulates plant development. Systemic signalling is a well known phenomenon in plants that are attacked by herbivores or pathogens (Leon *et al.*, 2001). It also plays a fascinating role in flower induction, where the leaves signal the environment and the shoot apical meristem is the responder (Reeves & Coupland, 2000; Mouradov *et al.*, 2002). It is only recently that long-distance systemic signalling has also been related to plant acclimation and leaf development (Karpinski *et al.*, 1999; Lake *et al.*, 2001).

### Future prospects

Current technologies in plant signalling are numerous, but unfortunately often suffer from one or other major limitation. Patch-clamp analysis, for example, provides information about ion channel activities, but generally requires individual protoplasts to be released from the tissue environment. Therefore, the neighbourhood of the naturally occurring cells is not experienced under assay conditions. The activity of individual genes can be tested with cellular precision by promoter-reporter gene fusions, *in situ* hybridization, or single-cell RT-PCR (reverse transcription, coupled to polymerase chain reaction; Brandt *et al.*, 1999),

but these methods typically only yield static images of the cell, or at best a scattered time resolution. Live imaging has been established, however, taking advantage of fluorescent probes and protein labels (such as green fluorescent protein; Ehrhardt, 2003). Recently, it has also become possible to study cellular events with a more global genomics view (Kehr, 2003). Thus, one can now measure the transcript levels of thousands of genes in just a small number of cells. To do so, picolitre fractions of individual cells are carefully sampled with microcapillaries and the mRNA in it is then surveyed using array-based expression profiling (Brandt *et al.*, 2002). Similarly, individual cells or pools of cells can be sampled from embedded tissue sections by laser capture microdissection (LCM), providing DNA, RNA and protein for the profiling of genomic characteristics, gene expression and protein spectra (Kerk *et al.*, 2003; Nakazono *et al.*, 2003).

One of the limitations posed by the above and many related techniques is that the *interaction* of cells is very difficult to analyse. Therefore, technical improvements that target cellular communication are highly welcome. The nanoinfusion procedure described by Hanstein & Felle is a step in the right direction. It may be particularly fruitful to apply nanoinfusion to transgenic plants altered in signalling pathways, or combine it with any of the other tools established in molecular and cellular biology, such as transient gene over-expression, RNA interference, or chemical genomics. Simply imagine – we could modify the physiological status of one cell (for example by cell-selective RNA interference), monitor online what happens in the intercellular compartment (e.g. by nanoinfusion-type protocols), and detect what the neighbouring cells do (say by a protein that carries a fluorescent tag).

Recently, two other minimally invasive techniques aiming at a more integrated view of the plant have been reported. Herdel *et al.* (2001) developed a set-up to monitor, continuously, ion concentrations in the xylem sap under full control of nutrient supply. Wegner & Zimmermann (2002) established a multifunctional xylem probe to measure the K<sup>+</sup> concentration in individual xylem vessels together with xylem pressure and trans-root potential. Eventually, we may come up with even more ingenious technologies that should allow us to study the integration of short-distance and systemic communication pathways that trigger the developmental programming in response to environmental inputs.

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**Key words:** molecular signalling, leaf development, nanoinfusion, systemic signalling.

## Symbiotic signaling: new functions for familiar proteins

Mycorrhizal associations enhance net photosynthesis, offer the potential for increased below ground carbon sequestration under elevated carbon dioxide concentrations, and hold promise for phytoremediation (Lussenhop *et al.*, 1998; Fitter *et al.*, 2000; Rufyikiri *et al.*, 2002). Despite all this, very little is known about the mechanistic details of host-symbiont recognition, the pathways leading to symbiosis-specific development and subsequent nutrient exchange. The research presented in this issue by Sundaram *et al.* (pp. 525–534) – showing the results of yeast two-hybrid screens identifying protein partners interacting with a symbiosis-regulated Ras-like protein, *Lbras*, from the ECM fungus *Laccaria bicolor* – is therefore a significant step forward.

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*'Ras proteins are involved in a dizzying array of regulatory activities in fungal systems including nucleotide and carbohydrate metabolism, cell division and DNA synthesis, pathogenicity, defense, membrane organization, cell death and aging'*

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### Symbiotic signaling in a broad context

The mycorrhizal fungi are thought to have played a significant role in the earliest evolution of land-based plants based on fossil records at least 460 million yr old with indications that their origins may be twice as old (Redecker *et al.*, 2000; Schussler, 2002). Today a few hundred species of (AM) fungi colonize more than a hundred thousand different species of plants, creating a mutually beneficial biochemical economy (Smith & Read, 1997). The lack of specificity in AM partnerships contrasts with the exquisite specificity of the *Rhizobium*–legume symbioses (Soltis *et al.*, 1995; Hirsch *et al.*, 2001; Doyle & Luckow, 2003). Ironically, several plant mutations affecting early steps in symbiotic signaling with rhizobia are also defective for mycorrhizal colonization, suggesting the nitrogen-fixing symbiosis evolved utilizing pre-existing pathways required for AM symbiosis. One example is the receptor kinase-like proteins identified in pea and *Lotus* that are members of a large family of plant and animal receptors containing

an extracellular leucine-rich motif (Stracke *et al.*, 2002; Endre *et al.*, 2002). Another plant gene required for symbiosis, the Sym4 protein from *Lotus* (Bonfante *et al.*, 2000), appears to exert its effects through changes in the cytoskeleton mediating compatibility responses in the epidermal tissues of the plant host (Genre & Bonfante, 2002). These findings and others underscore the importance of studying ECM and AM symbiotic signaling in the broadest possible context in order to increase our understanding of the biochemistry and ecology of these organisms (Martin *et al.*, 2001).

### Fungal regulatory genes

The search for ECM and AM fungal regulatory genes is beginning to bear fruit, as the work of Sundaram *et al.* demonstrates. Lbras was first identified in an EST screen and the full length *Lbras* was shown to possess several remarkable characteristics, including the ability to complement a yeast Ras2 mutant and transform mouse embryonic stem cells, altering their growth rate and morphology to an oncogenic phenotype (Sundaram *et al.*, 2001). Expression of the single copy *Lbras* requires interaction with host root factors with transient expression observable within 6 h and stable transcription after 24 h. The protein is localized within membranous regions of the Hartig net and mantle particularly along the periphery of the cell. It is not yet known if *Lbras* is modified by acylation events as occurs with other Ras proteins. Interestingly, while the *Arabidopsis* genome encodes many genes belonging to the Ras superfamily (Rab, Rho, Arf, and Ran) there are no true *ras* genes to be found (Vernoud *et al.*, 2003).

Ras proteins are involved in a dizzying array of regulatory activities in fungal systems including nucleotide and carbohydrate metabolism, cell division and DNA synthesis, pathogenicity, defense, membrane organization, cell death and aging. In yeast, the *Ras2* gene encodes a homolog of the mammalian oncogene *RAS* and is highly related to the yeast *RAS1* gene (Kataoka *et al.*, 1984). Ras2p is a small GTP-binding protein localized to the yeast plasma membrane as a result of the modification of its C-terminus with palmitoyl and farnesyl groups (Bhattacharya *et al.*, 1995). Ras2p regulates processes such as sporulation, filamentous growth and the nitrogen starvation response through its effects on yeast adenylate cyclase (encoded by the *CYRI* gene). In the activated, GTP-bound form Ras2p directly stimulates the production of cAMP by adenylate cyclase (Broek *et al.*, 1985). Cdc25p binds to and activates Ras2p by directly stimulating the exchange of GDP for GTP (Lai *et al.*, 1993). Conversely, the redundant proteins Ira1p and Ira2p inactivate Ras2p by stimulating hydrolysis of GTP to GDP (Parrini *et al.*, 1996). In the alfalfa fungal phytopathogen *Colletotrichum trifolii*, suppression of Ras activity significantly decreases fungal germination frequencies and hyphal growth rates (Ha *et al.*, 2003). Ras is also known to regulate spore germination in *Neurospora* and *Aspergillus* (d'Enfert, 1997) and is essential

for morphogenetic switching to hyphal growth and pathogenesis in *Candida albicans* (Rocha *et al.*, 2001). Three facts become abundantly clear from these examples:

1. Ras is a multifunctional protein regulating developmental pathways in free-living, symbiotic and pathogenic fungi.
2. Control of Ras activity depends not only on its pattern of expression, but also on factors that regulate Ras interaction with GDP (inactive) and GTP (active).
3. The key to understanding symbiosis-specific Ras regulation will be the identification of its protein partners.

### Ras-interacting yeast two-hybrid mycorrhizal clones

Three distinct *Lbras*-interacting gene products were identified by Sundaram *et al.* and designated RythmA, RythmB and Rythm C (Ras interacting yeast two hybrid mycorrhizal clones). The RythmB sequence showed no significant similarities to known genes and the RythmC sequence had weak similarity to a receptor kinase. Interestingly RythmA resembles the eukaryotic AP180 protein family. This family displays an Asn-Pro-Phe motif required for protein-protein interactions and several sites suggestive of phosphorylation sites for canonical casein kinase II, protein kinase C, and tyrosine kinases. The C-terminus of the RythmA protein contains a fungal hydrophobin motif as well, found in other fungal symbiotic proteins, although the functional significance of this observation is not yet clear.

Immuno-localization of *LBRAS* protein revealed a clustering in the vicinity of dolipore-septum regions in the Hartig net region. The dolipore allows the cytoplasm of adjacent ECM cells to mix, yet another novel feature of hyphal biology. It is tempting to speculate that *Lbras*-RythmA proteins are involved in the regulation of vesicle traffic in this region. If so, this would indicate that *Lbras* has an activity resembling plant and mammalian Rab GTPases that control vesicle assembly and transport (Sohn *et al.*, 2003). The probability that *Lbras* will manifest other regulatory activities is high, given the diversity of Ras functions in different organisms and the isolation of RythmB and RythmC from *L. bicolor* in the two-hybrid screen. The *Lbras* protein is most closely related to the *Aras* protein from *Aspergillus nidulans*, yet a BLAST search of the *A. nidulans* genome with the RythmA sequence failed to identify a gene product with any significant degree of homology. Thus, it seems likely that the genetic differences along the continuum of pathogenic and symbiotic fungi may be productively explored by focusing attention on novel proteins that interact with key common regulatory components.

### Perspectives

Future work will be needed to elucidate the mechanism of *Lbras* regulation and determine which other symbiotic

pathways are directly or indirectly regulated by this key protein. Critical questions include the issue of Ras-dependent regulation of cAMP levels, and whether the ECM utilize the MAP kinase signaling pathways as well. Analysis of the Lbras and RythmA promoters for *cis* and *trans* elements that regulate its symbiosis-specific expression should reveal components of the pathway that links ECM gene expression to diffusible plant root signaling compounds. Finally, given the nutrient exchanges that lie at the heart of ECM and AM fungal symbioses, an understanding of the mechanisms of long-distance inter- and intracellular transport will require understanding not just biochemical pathways but also the regulated movement of organelles and nutrient 'bodies' like glycogen and lipid bodies (Sohn *et al.*, 2003). The work of Sundaram *et al.* provides an important and exciting starting point for these investigations.

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**Key words:** symbiotic signaling, beneficial fungi, ectomycorrhizal (ECM) fungi, yeast two-hybrid screens, symbiosis-regulated Ras-like protein, *Laccaria bicolor*.

# Genotypic control and environmental plasticity – foliar physiognomy and paleoecology

Paleobotanists have likened the splitting open of sedimentary rocks and finding fossil plants contained within to the opening of Christmas presents. The anticipation, the awe, and the following pleasure and excitement all fuel the analogy – but it breaks down eventually, as the fossils come with neither a parts list nor the ever-important instruction manual. Paleobotanists are then left with major questions. What does the presence of this suite of fossils in the rock really mean? What inferences and conclusions can be made about the past vegetation based on the presence of these – and equally, the absence of other – plant remains? Paleobotany, as well as paleozoology, is in part driven by uniformitarianism – if even for no other reason than the fact that only in extant plants and animals can we observe and experiment with their environmental relationships. If one assumes that modern, observable, basic physiological and environmental principles can be applied to past vegetation, then it is possible to make inferences about those floras. It is into this arena that the paper by Hovenden & Van der Schoor (this issue, pp. 581–590) can be placed.

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*A fossil deposit is a wondrous thing – it can comprise layers of rock that each hold a 'freeze-frame' from the moving picture that is the evolution of life'*

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## Good news for paleoecologists

By elegantly testing the age old 'heredity vs environment' argument in the leaf characteristics of extant *Nothofagus cunninghamii* (Hook) Oerst. from Tasmania (Australia), Hovenden & Van der Schoor have not only provided excellent data on the growth and interpretation of this species through time, but have helped outline numerous clear signs (both warnings and guideposts) to other researchers in the fields of foliar physiognomy and general paleobotany. The specific aim of the paper is to test the relative contribution of genotypic control and environmental plasticity as it applies to some major (and frequently used) foliar physiognomic characters, including leaf length, area, thickness and stomatal density.

The authors cite past work as showing strong genotypic control of leaves in this species, but the 'good news' from their data (at least for paleoecologists) is that new results suggest that the environment shows a significantly greater effect in both leaf length and area, with thickness being the only character obviously more rigorously controlled by genotype. This last character is of little consequence to paleobotanical studies, in that only very rarely are fossils preserved in such a way as to allow total leaf thickness to be measured. The other important result presented in the paper (for paleobotanists again) is that this species supports the old adage elucidated by Bailey & Sinnott (1916) that 'the more tropical the flora, the larger the leaves' and the more recent corollary 'the higher the altitude, the smaller the leaves (and as Hovenden & Van der Schoor reiterate, the greater the stomatal density)'.

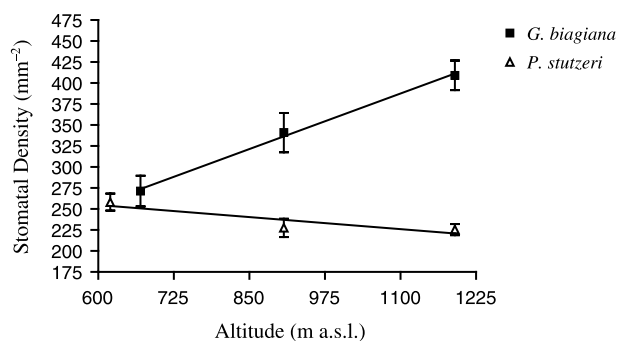
In illustrating the great potential value this paper has to the discipline of paleobotany, there are three areas upon which to focus:

- 1 The importance of species-specific studies, including an example of strong (and even inverse) results obtained from different species.
- 2 The importance of multiple life-forms in such studies.
- 3 The importance of the effect that dispersal of leaves prior to deposition may have on interpreting fossil deposits or, more specifically, the effect that leaves with an origin at higher elevation relative to their deposition may have on a physiognomic signature at that location.

## Will all relatives behave the same?

There are some instances in this natural world where one can take comfort in assuming that relationships with natural (and unnatural) processes are shared between closely related organisms. For example, it can be assumed that a deadly toxin known to be lethal to frogs in one stream will penetrate the porous skin of other frog species and kill them if their habitat also becomes polluted with this toxin. Frogs are frogs in this example and the species (or genus) will likely have little consequence on the observable outcome. For the most part such obvious broadly based assumptions can be considered safe hypotheses, but there always needs to be great care taken to make sure that you aren't giving too much ecological credit where it isn't due.

We would like to introduce a case from another Gondwanic family, the Cunoniaceae, where assumptions regarding the responses to change in environmental conditions cannot be shared among species. Recently collected data on two members of this family have shown greatly different morphological responses related to changes in environmental conditions associated with changing elevation (P. Gordon, unpublished). Specifically, these two species (*Geissios biagiana* (F. Muell.) F. Muell. and *Pullea stutzeri* (F. Muell.) Gibbs) show opposite linear relationships in stomatal density with increase in



**Fig. 1** Graph showing respective linear responses in stomatal density to changes in altitude for *Geissos biagiana* ( $y = 0.26423x + 96.8239$ ;  $r^2 = 0.663383$ ;  $P = 0.0002$ ,  $n = 5$ ) and *Pullea stutzeri* ( $y = -0.05731x + 289.0$ ;  $r^2 = 0.3079$ ;  $P = 0.0318$ ;  $n = 5$ ). Error bars show  $\pm$  SE.

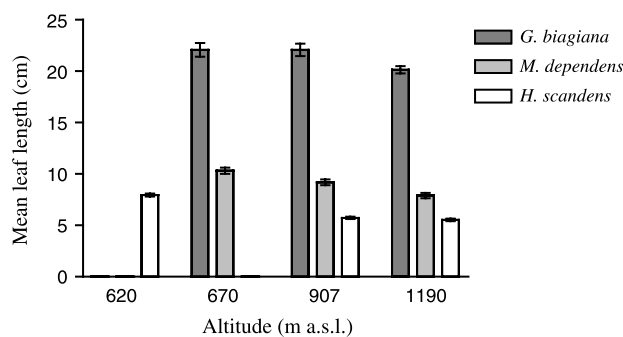
altitude (Fig. 1). This study was conducted on Mt Lewis, on the northern edge of the Atherton Tablelands in northern Queensland, Australia.

This relates to Hovenden & Van der Schoor's rightful assertion that there is strong evidence for the degree of environmental plasticity and adaptation being species dependant. Another example can be found in the Lauraceae (cinnamon family) where it has been shown that correlations between physiognomic features and the environment are predictable for *Neolitsea dealbata* (R.Br.) Merr. (Greenwood *et al.*, 2003). However, other taxa in this family (e.g. *Litsea leafiana* (F. Muell.) Merr.) do not show this trend (D. Christophel, unpublished).

With these studies in mind, we are compelled to make some cautionary comments directed towards palaeobotanists and paleoecologists who attempt to place extant species/environment relationships on extinct taxa of assumed close relation in order to obtain proxy paleoenvironmental data. With relatively little published in the area it is our opinion that the number of examples where there are found to be species-specific responses will only continue to grow. This possible confounding factor is one that needs close and careful consideration and the Hovenden & Van der Schoor paper helps to highlight the issue.

### Vines, herbs and shrubs

A fossil deposit is a wondrous thing – it can comprise layers of rock that each hold a 'freeze-frame' from the moving picture that is the evolution of life. What makes up this abstract picture of a past environment? The study of fossil plant deposits is a specific and important example of where this basic question needs to be asked. What is the habit of the plants from which the leaves, flowers, fruits and seeds come? It cannot be assumed that all leaves belonging to one species come from one plant, and it would be equally naive to assume that all leaves in the deposit came from only trees without any input from vines, herbs or shrubs. The study of



**Fig. 2** Graph showing the significant response in leaf length to changing altitude for the tree species *Geissos biagiana* ( $F = 13.42$ ;  $df = 2, 147$ ;  $P < 0.0001$ ) compared to the vines *Hibbertia scandens* ( $F = 92.50$ ;  $df = 2, 147$ ;  $P < 0.0001$ ) and *Maesa dependens* ( $F = 17.84$ ;  $df = 2, 147$ ;  $P < 0.0001$ ).

foliar physiognomy seems to be heavily biased towards tree species and there is little available literature describing the morphological relationships of other plant forms to their surrounding environmental conditions. While the target species of Hovenden & Van der Schoor's paper is also a tree species, they mention several relatively recent studies that focus on the foliar physiognomy of other life forms. This research is invaluable in helping to understand and decipher any proxy-related estimation of paleoclimate.

If differing plant forms are shown to have dissimilar morphological responses to environmental conditions, then it is crucial to take account of the relative proportion of these forms and factor this into the interpretation of a fossil flora – or at least to be aware of the different signatures that components may yield. The reality remains that except in a few obvious cases, unless the identity of fossil taxa can be determined, its life form may remain unknown. Some exceptions do exist, however, in that the presence of regular chordate bases, tendrils, or sharply bent petioles will often suggest vines.

Using the previously mentioned tree species (*G. biagiana*) and two vine species that are also common in the rainforest habitat of north-eastern Queensland (Australia), *Hibbertia scandens* (Wild.) Gilg and *Maesa dependens* F. Muell., we have compared the response in leaf length to increasing elevation (Fig. 2). Here it is seen that the two vine species chosen show very similar, significant reductions in leaf length with increasing altitude, as does the selected tree species. As previously mentioned, this fits with the generalization cited by Hovenden & Van der Schoor, and clearly suggests that plant forms such as vines can and do show very similar foliar physiognomic responses to the one commonly cited for trees. This group of plants, however, could well show species-specific rather than genus or family specific responses as found in trees, and further work is required.

While Webb's (1959) classification of the Australian rain forests was based in large part on the physiognomy of canopy



leaves, many of his forest types are named and qualified by the presence and/or frequency of woody vines, which implies their importance in the vegetation. The work of Greenwood (1992) in examining the physiognomic signatures of leaf litter from Webb's seminal localities, modified Webb's rainforest classification system to make it more useful to paleobotanists. Greenwood found a significant drop in average size for leaves in the litter of each of the rain forest types relative to the canopy. While he attributed much of it to taphonomic bias, we believe that some of that bias was likely caused by 'compositional' bias – for example, the litter being composed of more life forms. Quantification of this on a 'per species' basis is required to conform with the conclusions and suggestions found in the Hovenden & Van der Schoor paper concerning species-specific variations.

### Spacial and altitudinal dispersal: autochthany versus allochthany

A final aspect of Hovenden & Van der Schoor's paper that holds strong relevance to the field of paleobotany is their noting of the significant variation in the foliar physiognomic response seen in *N. cunninghamii* over the relatively small geographic scale of 15 km. The idea of long or even short distance dispersal of leaves before their final deposition has long been of concern to paleobotanists interested in reconstruction of paleoclimate (e.g. Hill, 1981). Mt Lewis, where the two studies illustrated earlier were conducted, covers approx. 25 km across its altitudinal gradient. Leaves of one highly distinctive taxon [*Stenocarpus davallioides* D. Foreman and B. Hyland (Proteaceae)] which occurs only in small stands at an altitude of 1190 m have been found along stream beds at 670 m, at least 7 km from the nearest tree (D. Christophel, personal observation). Thus paleoecological interpretation of the climate of a given flora must also consider the relative allochthany/autochthany of the plant specimens included.

The paper by Hovenden & Van der Schoor may therefore be seen as a very significant contribution, not only to the understanding of the biology of a very important Tasmanian tree species, but as an excellent example of the type of studies necessary for our ultimate understanding of the relationship between heredity and environment both in the contemporary scene and through time. The importance of this kind of research to the field of paleoecology cannot be overstated, and paleobotanists will continue to benefit from their and related future studies.

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**Key words:** genotypic control, environmental plasticity, foliar physiognomy, paleoecology, paleobotany.

## Analysis

# Symbiotic sequencing for the *Populus* mesocosm

**Sequencing the endomycorrhizal fungus *Glomus intraradices* and the ectomycorrhizal basidiomycete *Laccaria bicolor* – United States Department of Energy Joint Genome Institute (JGI)**

Access the DOE Joint Genome Institute at <http://www.jgi.doe.gov/>

Completion of the genome sequence of the model tree *Populus trichocarpa*, the first perennial plant to be tackled, will paradoxically prove to be a flagship project for symbiosis research – in its wake, the United States Department of Energy Joint Genome Institute (JGI) is now planning on sequencing several known *Populus* associates, the endomycorrhizal fungus *Glomus intraradices* and the ectomycorrhizal basidiomycete *Laccaria bicolor*. Why focus on organisms associated with *Populus*? Why these organisms? What do *G. intraradices* and *L. bicolor* have to contribute to our knowledge of the function of symbiotic fungi?

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*In the next few years, numerous fungal genomes are scheduled to be sequenced, owing largely to the Fungal Genome Initiative at the Whitehead Institute'*

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### Mycorrhizal genomics

In order to gain a predictive understanding of the complex biological systems that evolve from mycorrhizal interactions, a surge of studies based on functional genomics (large scale EST sequencing, cDNA array analysis of gene expression, proteomics) have allowed an assessment of the development and functioning of arbuscular endomycorrhizal (AM) and ectomycorrhizal (ECM) symbioses on a larger scale (Martin, 2001). If there is a basic repertoire of fungal symbiotic genes (which surely there is), it can be accessed only by comparing

whole genomes of saprobic (e.g. *Coprinus cinereus*) and pathogenic (e.g. *Magnaporthe grisea* and *Ustilago maydis*) species with mycorrhizal genomes. The availability of genome sequences from ecologically and taxonomically diverse fungi will not only allow ongoing research on those species, but will enhance the value of other sequences through comparative studies of gene evolution, genome structure, metabolic and regulatory pathways, and symbiosis/pathogenesis. One of the major strengths of rhizosphere studies for addressing these issues is that realistic ecological interactions can be investigated in a restricted micro or mesocosm under environmentally controlled conditions with organisms whose genomes have been completely defined (Phillips *et al.*, 2003) and/or genetically modified.

In the next few years, numerous fungal genomes are scheduled to be sequenced, owing largely to the Fungal Genome Initiative at the Whitehead Institute (Cambridge, MA, USA – <http://www-genome.wi.mit.edu/annotation/fungi/fgi/>). This initiative has proposed to sequence up to 44 fungal genomes that include well studied models important to human health, plant pathogens, as well as mutualistic species (e.g. the ectomycorrhizal *Paxillus involutus* and *Tuber borchii*). However, no mycorrhizal genomes have been sequenced yet, and none are on the final Whitehead list. The resolve of the JGI sequence onto the *Populus*-associated *G. intraradices* and *L. bicolor* is therefore especially welcome.

### *Populus* – the perennial favorite

A relatively small number of forest tree species have been subjected to intensive molecular genetic analysis. Trees in general are difficult experimental organisms, because of their large size and long generation times, and so attention has been focused on those species of greatest commercial importance, such as *Populus* and loblolly pine. Over the past decade, *Populus* has been advanced as a model woody plant because of its relatively modest genome size, extensive genetic resources, rapid early growth, ease of clonal propagation, and routine transformation protocols (Bradshaw *et al.*, 2000; Taylor, 2002; Wullschlegel *et al.*, 2002). The sequencing of the *Populus trichocarpa* genome to an approximately 7X depth adds to a long list of important attributes for research. Several groups in the USA, Canada, Sweden and France contributed significant technical resources, EST sets, fundamental *Populus* genome map information and data analysis to this international project (see the International *Populus* Genome Consortium site at <http://www.ornl.gov/ipgc/home.htm>). As a result of its

advanced silvicultural system, *Populus* has been developed for pulp and paper manufacturing, biofuels production, deployed for phytoremediation, and considered for its role in carbon management/sequestration settings (Tuskan, 1998; Tuskan & Walsh, 2001). The successful deployment of *Populus*-based systems for any of the abovementioned purposes depends not only on the ability of *Populus* as a genome to address the demands placed upon it by environmental stresses and stimuli unique to each scenario, but also by the suite of microorganisms that exist around and within the deployed genotypes. It is this consortium of endophytic symbionts that in part determines the effectiveness of the deployed tree system.

### **Populus as an ecosystem**

There is growing evidence that the microfauna and flora associated with a plant's rhizosphere influence the 'host' genotype's ability to respond to fluctuations in the environment (Daniell *et al.*, 1999; van der Heijden *et al.*, 1998; Staddon *et al.*, 2003). The same may be true for the hundreds of as yet unclassified endophytes: symbiotic, commensal and parasitic microorganisms that are continuously being discovered in the leaves and stems of *Populus* (G. Newcombe, unpublished). When viewed in total, a single large perennial plant – a tree – may be considered as an ecosystem in itself, where organisms interact beneficially or antagonistically over time, creating structure and function for further interactions to occur, to the point where the future of the host genotype lays in the balance. For example, the effectiveness of a *Populus* plantation on a contaminated site depends on the inherent ability of the *Populus* genotype to remove the contaminant from the soil and to transport the contaminant to the stem and leaves (Di Baccio *et al.*, 2003). The rate of transport is determined primarily by the transpiration stream, which is affected by mycorrhizal infection (Allen, 1991). This in turn affects overall water uptake and plant health, which influences susceptibility to leaf and stem pathogens, such as foliar rust fungi. Pathogens may ultimately kill the genotype and prevent timely site remediation if insufficient resistance responses occur.

Unlike ephemeral herbaceous annuals, it is the perennial nature of *Populus*, and other tree species, that evolutionarily facilitate the development of consortia of microorganisms that help shape responses to global climate change or stresses from environmental contaminants. The response of these systems to elevated CO<sub>2</sub> and temperature is virtually unknown, and could be an important component of overall ecosystem responses to climate change (Fitter *et al.*, 2000; Gielen *et al.*, 2002). Characterization of the *Populus* mesocosm would allow in-depth exploration of the coordinated community response to these abiotic stresses, thus adding a needed dimension to climate change research and providing

another step in the quest for mechanistic modeling of ecosystem responses.

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*'The challenge is to use the sequenced genomes to determine how mycorrhizal fungi evolve and function'*

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### **Glomus intraradices**

The AM symbiosis between fungi in the Glomeromycota and plants involves around two thirds of all plant species, and is of great ecological significance (Van der Heijden *et al.*, 1998; Daniell *et al.*, 1999; Fitter *et al.*, 2000). The key process in the symbiosis is the acquisition of the immobile phosphate ion from soil by the fungi, greatly enhancing plant P uptake (Smith & Read, 1997). The fungi are obligate symbionts, but there appears to be a low degree of specificity. The fungi may supply other nutrients (NH<sub>4</sub><sup>+</sup>) and may provide defense against pathogens, alter plant water relations and affect palatability to herbivores (Smith & Read, 1997). There are around 150 described species in the Glomeromycota, and about 200 000 plant species involved in the symbiosis. Despite the lack of specificity and the promiscuity of some of the fungi, the association is not in practice random (Sanders, 2003). Colonization of plants by mycorrhizal fungi results in a 5%–20% net increase in photosynthesis (Smith & Read, 1997). Thus AM fungi make a very large, if poorly understood, contribution to the global carbon cycling budget. The Glomeromycota have extremely unusual biological characteristics, being coenocytic, multinucleate, asexual, obligate symbionts with very wide host ranges. Arbuscular mycorrhizal fungi have strategic importance for environmental research (Fitter *et al.*, 2000). For example, between 4% and 20% more photosynthate goes to root systems in arbuscular mycorrhizal plants than in nonsymbiotic plants and a substantial fraction of photosynthate allocated below-ground is transferred to the fungus (Smith & Read, 1997). Because the large majority of land plants are AM and because net photosynthesis of mycorrhizal plants is higher than nonmycorrhizal plants, this represents a substantial fraction of photosynthetically assimilated carbon worldwide (Fitter *et al.*, 2000). There is also experimental evidence that when CO<sub>2</sub> levels are elevated, significantly more carbon is likely to move below ground under the influence of the AM symbiosis, and it may be that this will have a significant role in carbon

sequestration as global CO<sub>2</sub> levels rise (Lussenhop *et al.*, 1998). Mycorrhiza are also important for heavy metal remediation (Rufyikiri *et al.*, 2002). From an evolutionary standpoint, the AM fungi are unique obligate symbionts with coenocytic hyphae (lacking cellular structure) that transport organelles and nutrients over long distances. The regulation of gene expression in such a system with multiple nuclei migrating long distances is completely unexplored. Further, the concept of an individual does not apply, raising substantial questions about the natural selection and population genetics of these highly unusual organisms (Kuhn *et al.*, 2001). There is no known sexual cycle in AM fungi, although anastomosis between hyphae has been described. However, examination of EST sequences from a *G. intraradices* germinating spore cDNA library revealed three cDNAs with significant homology to fungal meiosis-specific proteins (Jun *et al.*, 2002). This raises intriguing questions and suggests there is much still to learn about the basic biology of AM fungi.

Genome sequencing of *G. intraradices* will have a tremendous impact on the scientific community as this, together with *L. bicolor*, will be the first mycorrhizal fungi to be sequenced. While no individual species is ideally suited to be a representative of an entire phylum, a number of factors make the choice of a model species particularly attractive for the AM fungi: there is a small number of species; the distinguishing characteristics of structure, life cycle, and life style are common to all AM fungi; and individual species such as *G. intraradices* occupy very wide ranges of hosts and ecosystems. Thus it may reasonably be concluded that insights and tools obtained from the genome sequence of *G. intraradices* will be very widely applied in AM research.

*G. intraradices* is a widespread mycorrhizal fungus found in different ecosystems throughout the world, including temperate and tropical locations. As a symbiont, *G. intraradices* is highly effective in mobilizing, taking up and transferring mineral nutrients from soils to plants, and it readily colonizes many plant species including agriculturally important species such as maize, wheat, alfalfa, rice, and key model plants such as *Medicago truncatula*, *Lotus japonicum*, and, most importantly in this context, *P. trichocarpa*. For these reasons *G. intraradices* is among the most studied AM fungi and is the prime ingredient in several commercially available inocula. As a representative of the AM fungi (Glomeromycota) *G. intraradices* is a close relative of two other highly studied AM fungal species: *G. etunicatum* and *G. mosseae*. *G. intraradices* can also be grown *in vitro* in dual culture with transformed carrot roots, and of particular importance is the development of a split plate system in which a separate fungal compartment allows a range of manipulations and observations to be made on the extraradical mycelium in the absence of other organisms (St.-Arnaud *et al.*, 1996). *G. intraradices* is also the only species whose spores are available commercially in pure form in large

quantities (Premier Tech, Quebec Canada), which is a resource for researchers worldwide and is mandatory for sequence-quality DNA preparation and construction of BAC libraries.

*G. intraradices* has a very small genome of *c.* 11–12 Mbases (I. Sanders, U. Lausanne, pers. comm.). In addition, random genomic survey sequencing (GSS) in the Lammers lab suggests a gene density comparable to, or higher than, yeast. Nearly 600 000 bp of GSS is now available, spread among 680 sequences (<http://darwin.nmsu.edu/~plammers/>). Fully 24% of these sequences yield a high quality BLAST match to the nonredundant GenBank databases. Analysis of the GSS data did not reveal any highly repetitive sequences that might make assembly of random shotgun sequence data into draft assemblies a difficult task. Nearly 3000 EST sequences from *G. intraradices* have been deposited in GenBank dbEST, along with nearly 1500 from other *Glomus* species (Jun *et al.*, 2002). Biolistic transformation has been achieved for AM fungi (Harrier & Millam, 2001), and efforts are under way to develop protocols for *G. intraradices* (Y. Shachar-Hill, pers. comm.).

### *Laccaria bicolor*

*Laccaria bicolor* is a member of the Tricholomataceae, a large order of ectomycorrhizal and saprobic basidiomycetes. The order is evolutionarily significant because switches between saprobic and ectomycorrhizal lifestyles have occurred several times within the order (Hibbett *et al.*, 2000). *Laccaria bicolor* is a common ectomycorrhizal fungus (ECM) that is a mutualist with many northern temperate forest trees including populus. It provides a useful system for studying the evolution of host and ecological specificity (Kropp & Mueller, 1999). In nature and in the laboratory, *L. bicolor* usually grows as a heterokaryon composed of two different nuclei, each of a different mating type, but axenic haploid strains isolated from spores are available (Di Battista *et al.*, 1996). Completion of the sexual cycle in the laboratory is possible (Godbout & Fortin, 1990). *Laccaria* has been used extensively in both basic and applied research. The physiological ecology of *L. bicolor* is well studied among ectomycorrhizal taxa, because it grows rapidly in culture and its mycorrhizas are easily established with tree roots under laboratory conditions (Kim *et al.*, 1998). *L. bicolor* is commonly used in microcosms and *in vitro* experiments in dual culture with *Populus* or conifer seedlings (Tagu *et al.*, 2001). These systems have been used to study carbon metabolism, nitrogen and phosphorous acquisition and transport, and the ability of this fungus to scavenge nutrients from soil. Finally, this species is used in large-scale commercial inoculation programs in forest nurseries worldwide to enhance growth of tree seedlings (Selosse *et al.*, 2000).

The haploid genome size of *L. bicolor* is estimated at *c.* 25 Mb, in the range of other basidiomycetes, such as

*Paxillus involutus* (20 Mb, Le Quéré *et al.*, 2002), *Ustilago maydis* (20 Mb), and *C. cinereus* (38 Mb). The gene density is estimated to be one gene every 2.5 kb (F. Martin, unpublished). Several cDNA libraries of vegetative mycelium grown under different growth conditions (e.g. N-depleted and C-rich media) (Peter *et al.*, 2003) and from *Populus* ectomycorrhizas (A. Kohler & F. Martin, unpublished) have been constructed. Currently, there is sequence from approx. 2000 ESTs and random genomic fragments deposited in GenBank dbEST (Podila *et al.*, 2002; Peter *et al.*, 2003). Ongoing EST projects (see EctomycorrhizaDB at: <http://mycor.nancy.inra.fr/ectomycorrhizadb/index.html>) will increase the number of sequences available for genome annotation. The 5000, 4000 and 2500 ESTs from *H. cylindrosporium* (H. Sentenac & D. Wipf, unpublished), *P. involutus* (T. Johansson, unpublished) and *Pisolithus microcarpus* (Peter *et al.*, 2003), respectively, could likely be used for annotation. A cosmid library, a partial genetic map, cDNA arrays and transformation systems are also available (Bills *et al.*, 1999; Podila *et al.*, 2002; Peter *et al.*, 2003). As a result, it should be possible to identify a gene of interest, alter its expression, and test the impact on biology and symbiosis formation. Comparison of the genomes of the different plant fungal pathogens with the *Laccaria* genome will be of interest to a wide range of genome and evolutionary scientists outside those working directly on this organism. The comparative genomics of *Laccaria* and *Glomus* will provide critical insights into the genetic makeup of free living vs obligate symbiotic fungi, insights into host range limitations in the ectomycorrhiza, comparisons of metabolism and nutrient exchange, and facilitate the study of host/symbiont signaling processes as well. Comparative genomics between *L. bicolor* and *G. intraradices* will also provide evolutionary and functional genetic clues about the endo- and ectomycorrhizal habits.

There is a network of cooperating laboratories working on *L. bicolor*, *P. involutus*, *Pisolithus* spp., *A. muscaria* and *Tuber borchii* that have organized efforts to share sequence information within the International Ectomycorrhiza Genome Consortium (<http://mycor.inra.nancy.fr/IEGC/>). The *Laccaria* sequence will be of broad interest to fungal researchers and to biologists working on plant–microbe interactions. Groups in the United States, UK, Sweden, France and Germany work on *L. bicolor* and related *Laccaria* species. The overall purpose of the initiative is to build on available resources and to provide a comprehensive understanding of the symbiotic process and related fungal biology and ecology.

## Outcomes

What will the whole genome sequences of *G. intraradices* and *L. bicolor* bring? Together with the *Populus* genome sequence, researchers will have in hand the genetic blueprints for the mycobionts and their host tree. This will

provide the ability to take a holistic approach in understanding how the symbionts interact with the tree host. We have already taken substantial steps in this direction. Combined genomics research on *Populus* and its associated mycorrhizal symbionts, together with the Fungal Genome Initiative, will not only allow the complete genetic blueprints of important fungal species to be determined and compared, but also to greatly accelerate such research on plant–microbe interactions. It should provide a platform for detailed comparative genomic analysis across the fungal taxa, including a comparison of saprotrophic, pathogenic and mutualistic species. The challenge is to use these sequenced genomes to determine how mycorrhizal fungi evolve and function.

Given the diversity within the mycorrhizal lineages, the availability of the *G. intraradices* and *L. bicolor* genome sequences should fuel interest in the study of additional symbiotic fungal genomes. EST programs of mycorrhizal fungi (Peter *et al.*, 2003) have revealed a large proportion of unknown sequences, so-called orphans, which might represent genes that rapidly diverge between closely related species. Defining their functions is a major undertaking, and any functional clues from sequence comparisons will help guide experimental design for studying their functions. Whole genome availability will certainly allow an in-depth analysis of these rapidly evolving genes that may code for specific functions, such as symbiosis. Analysis of this wealth of information is certain to provide breakthroughs in understanding of the molecular and cellular mechanisms involved in the development and biochemical pathways in symbiotic partners. In addition, it will allow us to answer fundamental questions about whether parasitic and symbiotic habits evolved through gene acquisition and loss, or gene regulation. The promoter analysis of the current compendium of mycorrhiza-regulated genes will provide the basis for a more precise molecular dissection of the complex genetic networks that control symbiosis development and function. Determination of entire genome sequences, however, is only the first step in understanding the inner workings of an organism. The next critical step is to elucidate the functions of these sequences and give biochemical, physiological and ecological meaning to this information. This will require an efficient integration of bioinformatics tools and genome-wide functional analyses, including gene disruption, transcriptomics and proteomics, to determine gene function (Tunlid & Talbot, 2002).

The *New Phytologist* Trust is organizing a symposium (October 2004 – see <http://www.newphytologist.org/popgen/>) for plant genomics researchers to explore applications of the *Populus* genome sequence and gain insights into the molecular bases of adaptation in natural populations. Genes are key players in ecological function, and genome-wide identification of plant and fungal genes that are transcribed in response to alteration in trophic webs within symbiotic associations and in the rhizosphere offers ecologists optimum

opportunities to understand soil and rhizosphere ecology. *Populus* eco-genomics will deepen our understanding of tree biology, particularly the genetic responses to stresses, pests and the environment. It may also provide insights and potential solutions to protecting and maximizing the value of forest ecosystems, hopefully leading to new sustainable strategies for breeding, nurturing and better utilization of trees.

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## Meetings

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### Ancient DNA – unlocking plants' fossil secrets

**Introducing genetic and palaeogenetic approaches in plant palaeoecology and archaeology, Bordeaux, France, September 2003**

Disillusion rather than enthusiasm had predominated among scientists after the first reports of successful but sometimes hardly repeatable retrieval of ancient DNA (aDNA) from very ancient specimens such as Miocene fossil leaf samples or even dinosaur eggs. In the past few years, however, scrupulous studies including a suite of controls for authenticity have provided the basis for regained confidence in the field of palaeogenetics. Furthermore, it has now been shown that animal and especially plant aDNA is not only present in fossil tissues but may even be indirectly retrieved from secondary samples such as fossil faeces (Poinar *et al.*, 1998; Poinar *et al.*, 2001) or sediments (Willerslev *et al.*, 2003). The recent Bordeaux symposium on the use of plant aDNA provided a forum for this renewed enthusiasm and illustrated the substantial progresses that are currently being made. A particular focus was on the main European forest tree species during the Quaternary period – using aDNA as a link between phylogeographic and classical palaeoecological reconstructions (the topic of the FOSSILVA project, which brought together teams of geneticists and pala-

eoecologists, coordinated by Jacques-Louis de Beaulieu (CNRS – Marseille, France)).

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*'Retrieving DNA information from well preserved Quaternary fossil material is no longer a dream'*

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### Reconciling neocology and palaeoecology

Until recently, reconstructing past plant population dynamics has relied either on studies of genetic variation based on contemporary populations or on the fossil record. The two approaches are somewhat discordant. Neocologists can investigate all aspects of an organism's phenotype and genotype but have no direct access to population dynamics (although recent developments, such as the availability of population-based DNA sequence data, and approaches, such as phylogeography (Avice, 2000) and the coalescent (Kingman, 1982), have introduced a temporal dimension that was missing in former equilibrium-based analyses). Palaeoecologists, on the contrary, have direct access to 'time', but can rely only on phenotypes, and usually incomplete ones. Nevertheless, as a result of some of the first truly interdisciplinary efforts between

the two groups (Cwynar & MacDonald, 1987; Comps *et al.*, 2001; Petit *et al.*, 2002, 2003) our understanding of postglacial migration in plants has been rapidly improving. In May 2003, another conference organised by the Royal Society in London on the evolutionary legacy of the ice ages allowed disparate groups to examine the degree to which the alternation of cold and warm stages during the Quaternary had affected the evolution of both plants and animals. These and other recent advances have contributed to an emerging synthesis on the evolutionary significance of the ice ages.

### Combining genetic and palaeoecological data

In recent years the use of pollen and macrofossil databanks has contributed significantly to a continuous increase in our knowledge of past plant distribution (Elenga *et al.*, 2000; Litt *et al.*, 2003) and ecology (Bennett, 1997). In Bordeaux, Eric Grimm (Illinois State Museum, USA) showed recent development achieved with the Global Pollen Database (GPD), which currently contains Quaternary pollen data from the Americas, Europe, Africa, Asia, and the Indo-Pacific regions. The objective is to assemble data from fossil deposits and modern surface samples into a relational database and to make them readily available to the scientific community (<http://www.ngdc.noaa.gov/paleo/gpd.html>). Broad-scale palaeovegetation patterns inferred from such mapped pollen data have largely confirmed that plant communities are impermanent assemblages of taxa and often have no modern analogues, as first argued by Davis (1976). Simultaneously, during the past 10 yr, population genetics based on modern DNA techniques has shed new light on the postglacial migration history of many temperate plants. At the symposium, Giovanni G. Vendramin (CNR, Florence, Italy) presented an example of such studies. An impressively large dataset of the genetic variation in European broadleaved and coniferous species had been assembled using chloroplast markers and compared to pollen analytical data. The results showed how several temperate tree species had been similarly affected by successive ice ages and by the dissected geography of southern Europe, despite responding individually in terms of timing and routes of expansion (Petit *et al.*, 2003). In particular, genetic diversity is higher in the central part of the range than in the Mediterranean part for a majority of the trees and shrubs investigated, even though the latter region had clearly provided areas suitable for long-term refugia. Presumably, admixture had occurred at intermediate latitudes at the confluence of the various colonisation routes emerging from the Mediterranean peninsulas. The presence of plant refugia in central and southern Europe during the last glacial maximum has long been a matter of debate, which inevitably highlights one of the main shortfalls of the use of fossil pollen to reconstruct past vegetation history: pollen found at a certain site can represent both local and long-distance dispersal by wind. This limits the spatial resolution of the method, especially when the pollen accumulation rate

is slow, as was the case during the last glacial maximum. Hence, other analytical methods should be sought, such as analyses of plant macrofossil assemblages from lake sediments and of subfossil wood preserved in the record as macroscopic charcoal.

An example where all three proxies (genetic markers, pollen and macrofossils) had been integrated was provided for European beech (*Fagus sylvatica*) by Donatella Magri (University La Sapienza, Roma) and colleagues from the FOSSILVA project. Pollen data from several hundred lake and mire cores, mostly retrieved from the European pollen database, were combined with more scattered, but well-dated, macrofossil data and with an extensive genetic dataset obtained from over 600 forests distributed throughout the species range. The synthesis suggested a new scenario for postglacial recolonization by beech, and indicated that colonisation events predating the last glacial maximum need to be taken into account to explain the contemporary genetic patterns of this species. Obviously, questions remain and further confirmation will be needed that might necessitate resorting to aDNA.

### Ancient DNA – potential and pitfalls

A major constraint remains for population geneticists, who must extrapolate from modern molecular data. However, retrieving DNA information from well-preserved Quaternary fossil material is no longer a dream and should therefore allow this limitation to be bypassed. The survival of aDNA in specimens up to several thousand years old is now well established, and retrieving DNA from even older remains of the late Quaternary (up to 100 000 yr ago) has added further insights to the study of many evolutionary processes (Krings *et al.*, 1997; Poinar *et al.*, 1998; Leonard *et al.*, 2000; Cooper *et al.*, 2001; Barnes *et al.*, 2002; Lambert *et al.*, 2002).

Unfortunately, for various reasons, studies of plant aDNA have lagged behind those of animals and humans. Despite the wealth of plant subfossil material available for molecular study, from wood to leaves and needles or even single pollen grains (Suyama *et al.*, 1996; Deguilloux *et al.*, 2002, 2003; Ziegenhagen *et al.*, 2003), aDNA appears to be retrievable in only a small fraction of the samples investigated. Furthermore, because of the minute amounts and degraded nature of aDNA there is a constant risk of contamination. The need for authentication became clear in the mid-1990s when a number of high-profile studies were shown to be non-reproducible, and in successive years standard criteria had to be developed to determine the ancient origin of DNA sequences (Handt *et al.*, 1994; Cooper & Poinar, 2000) (Box 1).

### DNA preservation in ancient specimens

One important property of aDNA is its fragmented nature, mostly caused by the hydrolysis of the DNA phosphodiester bonds and the *N*-glycosyl bonds. Typically, only a small proportion of fossil specimens contain DNA that can be amplified



**Box 1** Authenticity of ancient DNA sequences

The extraction and preparation of the PCR required for amplification of ancient DNA (aDNA) must be done in a laboratory that is rigorously separated from work involving modern DNA, equipment should be regularly treated with bleach and UV irradiation, and protective clothing should always be worn (Handt *et al.*, 1994; Höss *et al.*, 1994). In addition to these basic lab procedures, a number of criteria have been developed to determine the ancient origin of DNA sequences (Cooper & Poinar, 2000):

- **Physically isolated work area** To avoid contamination, it is essential that, before the amplification stage, all aDNA research is carried out in a dedicated, isolated environment. A building in which large amounts of the target DNA are routinely amplified should be banned.
- **Control amplifications** Multiple extraction and PCR controls must be performed to detect sporadic or low-copy number contamination, although carrier effects do limit their efficacy. All contaminated results should be reported, and positive controls should generally be avoided, as they increase contamination risk.
- **Appropriate molecular behaviour** PCR amplification strength should be inversely related to product size (large 500- to 1000-bp products are unusual) and sequences should make phylogenetic sense.
- **Reproducibility** Results should be repeatable from the same, and different, DNA extracts of a given specimen.
- **Cloning** Direct PCR sequences must be verified by cloning amplified products to determine the ratio of endogenous to exogenous sequences, or damage-induced errors. Overlapping fragments are desirable to confirm that sequence variation is authentic and not the product of errors introduced when PCR amplification starts from a small number of damaged templates.
- **Independent replication** Intra-laboratory contamination can only be discounted when separate samples of a specimen are extracted and sequenced in independent labs.
- **Biochemical preservation** Indirect evidence for DNA survival in a specimen can be provided by assessing the total amount, composition, and relative extent of diagenetic change in amino acids and other residues.
- **Quantitation** The copy number of the target DNA should be assessed using quantitative RT-PCR. When the number of starting templates is low (< 1000), it may be impossible to exclude the possibility of sporadic contamination, especially for human DNA studies.
- **Associated remains** Evidence that DNA has survived in associated material from other species is critical supporting evidence. Such remains could also make good negative controls for PCR amplifications of the target species.

by PCR. In such cases, rapid screening methods can be useful to identify the large fraction of samples that are so badly preserved that there is little hope of retrieving any aDNA. In particular, amino acid analyses (counts of the total amount of amino acids preserved in a specimen, amino acid composition and extent of racemization) have proven to be very useful proxies for monitoring DNA preservation in fossil samples (Poinar *et al.*, 1996). Results from such studies have suggested that the DNA preservation is mainly linked to the temperature and its constancy at a site, rather than to its age. An additional useful way to check the quality of old DNA sequences is the estimation of the number of template DNA molecules from which the PCR starts. Robert Blatter (Max Plank Institute, Leipzig, Germany) presented two studies, on 100-yr-old museum bones from 42 orang-utans and from 20 000-yr-old ground sloth coproliths, where different fragments of the mitochondrial rDNA region were quantified by RT-PCR to identify specimens suitable for population genetic studies. These methodologies are very promising and can be adapted to studies of other specimens, including plants.

### New uses for ancient DNA

An intriguing potential use of aDNA research is in revealing the genetic record of temporal changes in populations over millennia time-scales. So far, genetic changes within populations

have been reconstructed using only current patterns of genetic diversity. Given the dynamics of climate change and migration within continents, extrapolations based on such data are difficult. Ancient DNA can provide a direct record of the tempo and mode of genetic change within populations, and thus a means of testing and refining existing population models. The deposits with the largest potential are probably those of the Arctic permafrost, of high altitude caves, and of other cold and arid environments. Unfortunately, however, opportunities for these types of studies on aDNA are offered only when a sufficient number and distribution of fossil samples are available. As an example, in a recent study on fossil bones of Adélie penguins from Antarctica, Lambert *et al.* (2002) succeeded in measuring the rate of nucleotide evolution in the mitochondrial DNA by analysing an adequately large number of fossil samples. This study was possible because of the particular aspect of the life history of the animals studied and of the extreme environmental conditions in which the bones had been preserved. In plants, the retrieval and possibilities for analysis of well preserved fossilised hard tissues such as wood are very rare compared to the retrieval of bones in animals. Moreover, as explained at the symposium by Birgit Ziegenhagen (Phillips University of Marburg, Germany), the molecular analysis of woody, sclerenchymatic or dry tissues from plants is methodologically very difficult, even when the tissues are sampled from extant specimens. In this context, Yoshihisa

Suyama (Tohoku University, Sendai, Japan) presented a new and promising approach for the analysis of plant aDNA. Together with Laura Parducci (Uppsala University, Sweden), they have recently developed a method for the amplification and sequencing of short chloroplast DNA regions from single pollen grains of conifers isolated from Holocene lake sediments. If reproducible and applicable to different plant species, the method will allow direct estimates of the population dynamics in space and time and could reveal new details of the historical patterns of evolutionary change in plant species.

### Ancient DNA extracted from soil

Recently, a team at Copenhagen University (Denmark) led by Eske Willerslev and Anders Hansen succeeded in extracting DNA from Siberian permafrost sediments (Fig. 1) and from soil sampled in temperate caves from New Zealand, ranging in age from 10 000 yr to 400 000 yr (Willerslev *et al.*, 2003). As Willerslev explained in Bordeaux the team was initially looking for bacterial DNA but found out that they could recover fragments of plant chloroplast DNA in soil samples from permafrost cores. Eventually they were able to identify DNA sequences from at least 19 different plant taxa (angiosperms, gymnosperms and mosses) as well as from various animals including mammoth, bison and horse. Much of the plant DNA probably derived from roots, which would have been well protected under the frozen ground. The data presented suggested dramatic changes in the taxonomic diversity and composition of Beringian vegetation during the Quaternary. Perennial, dry, temperate cave sediments in New Zealand also yielded sequences of extinct biota, including two species of ratite moa and 29 plant taxa characteristic of the prehuman environment. Importantly, the latter result demonstrated that DNA, under particular

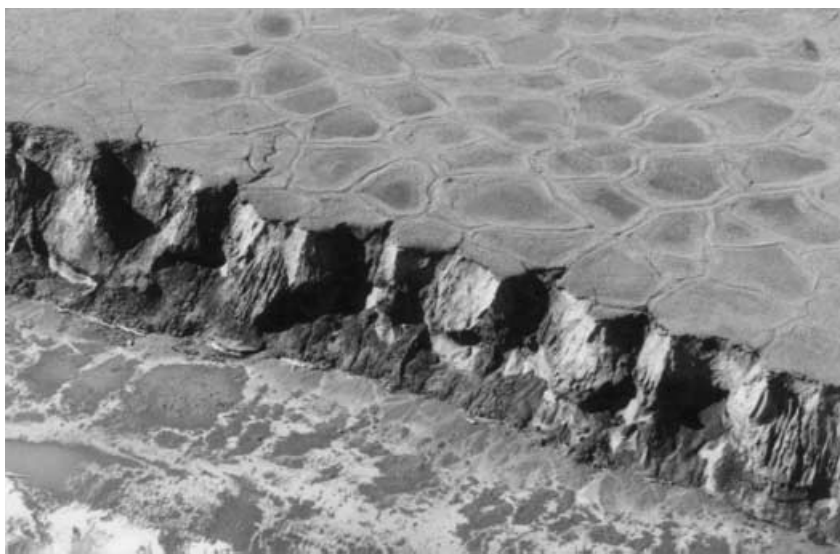
conditions such as dry environments, could be preserved even in unfrozen conditions.

### Perspectives

In plants, strict adherence to all the criteria for the authentication of aDNA is difficult and, in some cases, impossible to achieve – this was clear from many of the studies presented at Bordeaux. For example, the criteria of reproducibility, biochemical preservation and DNA quantitation are not applicable when studying single-pollen grains of plant specimens, because the fossil material is depleted with the first PCR analysis. Nevertheless, it is critical that the highest possible standards are applied if plant aDNA research is to remain credible. The Bordeaux meeting has highlighted the power of aDNA analysis as a technique – exemplified by the cross-over between paelaeogenetics and paelaeoecology, in which aDNA analysis is enabling new and sophisticated methods for hypothesis testing and model validation. The scientific rewards to both disciplines look set to follow the promising start of aDNA research.

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**Fig. 1** Siberian permafrost, showing an exposed permafrost brink at one of the key sites for permafrost drilling (Kolyma lowland, Yakutia, in the Siberian far east). The tundra above the brink shows polygon formations typical for permafrost areas. The exposed cliff, approx. 30 m high, represents hundreds of thousands of years of accumulation of frozen soil. Courtesy of David Gilichinsky and Eske Willerslev.

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