An inconvenient truth

In February this year, the Oscar award for the best documentary film went to An Inconvenient Truth. The film was based on a lecture by former US Vice President Al Gore on the perils of global warming and is already the third-highest grossing documentary ever. It is to be hoped that the message within reaches an ever wider international audience.

Seen as a complex statement, the title of the film has relevance in all walks of life, including research in plant science on the impacts of global change. Increasing concentrations of CO₂ in the atmosphere can drive complex changes in climate beyond the undesired warming potential of CO₂ itself. However, an inconvenient truth is that, in experiments, increasing concentrations of CO₂ are generally beneficial for plant growth, although with quite some variation among species and experimental designs (Körner, 2006). The simple plant perspective is that more CO₂ increases photosynthesis and reduces water loss, a double benefit (Ainsworth & Long, 2005), but with novel changes in CO₂-dependent gene expression still to be investigated in depth (Taylor et al., 2005). Further, CO₂ enrichment can reduce the impact of the pollutant tropospheric ozone (O₃) on plant productivity (King et al., 2005; Ashmore et al., 2006; Morgan et al., 2006), resulting in unique changes in gene expression that are found only when plants are exposed jointly to O₃ and CO₂ (Gupta et al., 2005). These experimental results clearly indicate a productivity benefit of rising CO₂ concentrations. Evidence that this is occurring naturally is less easy to obtain or to attribute unequivocally to rising CO₂. However, some long-term analyses of tree growth (Soulé & Knapp, 2006) show greater growth in the latter half of the 20th century, when CO₂ was rising most quickly. The growth responses also tended to be greater during drier years, as expected from CO₂ enrichment experiments, but it is inconvenient that these changes can not be ascribed with certainty to rising concentrations of CO₂ concentration.

Research on the influence of global change on plants growing in the field is dominated by the impacts of CO₂ enrichment, with the most natural conditions being present within Free Air CO₂ Enrichment designs. Unfortunately it is inconvenient to enrich plants to the gradual rate of increase of CO₂ observed in the atmosphere – that is the other human-controlled experiment that controlled systems are trying to predict. Instead, plants are treated to a step change in CO₂ concentration, probably no problem for plants starting from seeds, but a likely and unknown problem for existing plants, particularly those that are long-lived, where the persistence of pretreatment effects is poorly understood. Assessment of the impact of CO₂ enrichment on vegetation succession would be a natural way to identify the manner in which observed species-specific responses influence community composition, but few studies have adopted this approach (Dijkstra et al., 2002). The problems of securing long-term funding are a bothersome limitation to a more general application of such an approach.

The future survival of plant species is unlikely to be dominated by rising concentrations of CO₂ directly but rather by the negative impacts of climatic change, such as reductions in precipitation and extremes of temperature, where the beneficial effects of CO₂ enrichment may be less. Projections of the future changes, generally reductions of plant biodiversity, are based on models that are only concerned with changes in climate (Thomas et al., 2004). Any inconvenient impacts of CO₂ enrichment are not considered, although CO₂ experiments often demonstrate species-specific responses to CO₂, not only by higher plants (Körner, 2006) but also by mycorrhizal mutualists in the soil (Alberton et al., 2005). The many model simulations that address the impacts of changing temperature on vegetation are in direct contrast to a very small number of field-based warming experiments (Musil et al., 2005). Warming vegetation is difficult and inconvenient but necessary to understand the range of future species responses that may well be latitude or location specific.

There is a troublesome distance between practical experiments and the need to understand and predict the future responses of plants in the field. This has not prevented brave attempts to use empirical understanding within simulation models to predict future scenarios for plants in uncharted environmental conditions. However, models most generally agree closely in their simulations of the carbon and water cycles up to the present day but then begin to differ increasingly into the future (Cramer et al., 2001). It is an inconvenient truth that such uncertainty needs to be assimilated in some way (Midgley & Thuiller, 2005) for those policy makers that have the capacity to influence emission controls, but it’s difficult.

F. I. Woodward

Editor-in-Chief
The gas that opens gates: calcium channel activation by ethylene

The gaseous phytohormone ethylene is involved in the regulation of development and stress adaptation (Pierik et al., 2006). In some cases, ethylene has been proposed to act through elevation of cytosolic free \( \text{Ca}^{2+} \) ([\( \text{Ca}^{2+} \)]_cyt), for example in root hair elongation (Petruzzelli et al., 2003) and biotic stress gene induction (Raz & Fluhr, 1992). However, to date there has been no direct evidence for [\( \text{Ca}^{2+} \)]_cyt elevation by ethylene. In this issue of *New Phytologist* (pp. 507–515), an elegant biophysical study by Zhao and colleagues demonstrates that ethylene generation by ethephon or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) increases [\( \text{Ca}^{2+} \)]_cyt in tobacco (*Nicotiana tabacum*) suspension cells by activating a lanthanide-sensitive \( \text{Ca}^{2+} \) influx pathway. By applying patch clamp electrophysiology to protoplasts of the suspension cells, Zhao et al. were also able to identify a lanthanide-sensitive plasma membrane \( \text{Ca}^{2+} \) influx channel that is activated by ethephon or ACC and most probably underpins the [\( \text{Ca}^{2+} \)]_cyt elevation. Thus, this work not only establishes that ethylene has the ability to act through [\( \text{Ca}^{2+} \)]_cyt as a second messenger but also delineates a key part of the mechanism – the calcium channel.

Ethylene has been termed a “Janus” phytohormone as it is now becoming clear that it can mediate opposite effects on cell elongation; low concentrations stimulate, high inhibit.

References


Cramer W, Bondeau A, Woodward FI, Prentice IC, Betts RA, Brovkin V, Dijkstra P, Hymus G, Colavito D, Vieglais DA, Cundari CM, Johnson Gupta P, Duplessis S, White H, Karnosky DF, Martin F, Podila GK. 1997. Acid (ACC) increases [\( \text{Ca}^{2+} \)]_cyt in tobacco (*Nicotiana tabacum*) through [\( \text{Ca}^{2+} \)]_cyt as a second messenger but also delineates a calcium channel activation by ethylene. In this issue of *New Phytologist* (pp. 507–515), an elegant biophysical study by Zhao and colleagues demonstrates that ethylene generation by ethephon or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) increases [\( \text{Ca}^{2+} \)]_cyt in tobacco (*Nicotiana tabacum*) suspension cells by activating a lanthanide-sensitive \( \text{Ca}^{2+} \) influx pathway. By applying patch clamp electrophysiology to protoplasts of the suspension cells, Zhao et al. were also able to identify a lanthanide-sensitive plasma membrane \( \text{Ca}^{2+} \) influx channel that is activated by ethephon or ACC and most probably underpins the [\( \text{Ca}^{2+} \)]_cyt elevation. Thus, this work not only establishes that ethylene has the ability to act through [\( \text{Ca}^{2+} \)]_cyt as a second messenger but also delineates a key part of the mechanism – the calcium channel.

Ethylene has been termed a “Janus” phytohormone as it is now becoming clear that it can mediate opposite effects on cell elongation; low concentrations stimulate, high inhibit.

From receptor to channel

Ethylene receptors are now thought to reside in the endoplasmic reticulum (ER) rather than in the plasma membrane (PM). Zhao et al.’s finding that an antagonist of ethylene perception (1-methylcyclopropane (1-MCP)) prevented PM channel activation by ethephon or ACC means that there must be a signalling relay from the ER to the PM. What could that be? The clues may lie in the nature of the channel itself. The channel described by Zhao et al. is of a type similar to that characterized in primarily root cell (e.g. Véry & Davies, 2000; Foreman et al., 2003; Demidchik et al., 2007), pollen (e.g. Shang et al., 2005) and guard cell (e.g. Murata et al., 2001) PM. Briefly, all are voltage gated – they open to permit selective Ca2+ influx, in this case at very negative or ‘hyperpolarized’ resting membrane voltage, are permeant to barium (Ba2+) and are blocked by lanthanides. These hyperpolarization-activated Ca2+ channels (HACCs) have been found to be activated by a range of signalling agents including [Ca2+]cyt (Véry & Davies, 2000), extracellular calmodulin (Shang et al., 2005), H2O2 (Murata et al., 2001; Demidchik et al., 2007) and hydroxyl radicals (Foreman et al., 2003). Zhao et al. incorporated the divalent cation chelator ethyleneglycoltetraacetic acid (EGTA) and 0.1 mM dithiothreitol (DTT) as an antioxidant in their patch clamp pipette solutions, which could have prevented ethylene-induced elevation of [Ca2+]cyt and accumulation of intracellular reactive oxygen species (ROS) to activate the channels. It would be really useful in future studies to measure [Ca2+]cyt and ROS alongside the ethylene-induced HACC in protoplasts. Such measurements would be critical to establishing the sequential signal outputs of the ethylene receptors and positioning HACCs in this signal transduction pathway.

Arabidopsis contains five ethylene receptors, and binding to the ethylene receptor 1 (ETR1) receptor releases the restriction on signalling imposed by the Raf-like kinase constitutive triple response 1 (CTR1) (Fig 1). Downstream of CTR1 lies ethylene insensitive 2 (EIN2), an integral membrane protein related to the natural resistance-associated macrophage protein (NRAMP) family of divalent cation transporters (reviewed by Pierik et al., 2006). The membrane location of EIN2 is unknown and it appears incapable of metal transport, ruling it out as a means to release Ca2+ from internal stores (to stimulate HACC activity) or to permit influx itself. Downstream of EIN2, if Ca2+ were released from internal stores (ER, mitochondria, plastids or the vacuole), that elevation of [Ca2+]cyt could activate the HACC (Véry & Davies, 2000) or stimulate PM Ca2+-dependent NADPH oxidase activity. The latter possibility could help explain EIN2-dependent ethylene stimulation of the stomatal Arabidopsis thaliana respiratory burst oxidase homologue F (AtrbohF) NADPH oxidase that results in intracellular H2O2 accumulation and stomatal closure (Desikan et al., 2006). NADPH oxidase activity results in production of extracellular superoxide anions which can lead to extracellular H2O2 peroxide formation (Fig. 1). Peroxide can cross the bilayer or its entry can be controlled by flux through aquaporins (Bienert et al., 2007). Application of extracellular H2O2 activates the guard cell HACC which is thought to contribute to the [Ca2+]cyt signal for abscisic acid (ABA)-induced closure (Murata et al., 2001) but it is not known at which membrane face H2O2 acts. It will be of great interest to see whether ethylene-induced HACC activation holds in guard cells.

Switching off the signal

The missing steps between the ethylene receptors and HACC, plus the intersect points with ROS signalling (particularly downstream of other phytohormones such as ABA), now need to be identified. HACC could well be a much earlier target, activated without an increase in [Ca2+]cyt but allowing Ca2+ influx to activate the NADPH oxidase. Intracellular H2O2 could be generated independently of
the NADPH oxidase to activate HACC (Fig. 1). Recent
evidence suggests that ethylene might activate the PM H+-ATPase (Vreeburg et al., 2005); this would hyperpolarize the
membrane voltage to activate HACC. However, it is tempting
to speculate that at some point Ca2+ release from the ER is
involved in this or other ROS/[Ca2+]cyt signalling pathways, as accumulation of intracellular H2O2 would inhibit the ER
Ca2+ release channel (Klüsener et al., 1997), providing a
near way to start terminating the [Ca2+]cyt elevation (Fig. 1).

Ethylene as a stimulator, inhibitor and adaptor;
are channels involved?

Ethylene has been termed a ‘Janus’ phytohormone as it is
now becoming clear that it can mediate opposite effects on
cell elongation; low concentrations stimulate, high inhibit
(Pierik et al., 2006). The negative or positive elongation
response to ethylene will depend on the species, cell type,
cellular developmental state, nutrient state, oxygen avail-
ability, light intensity and quality and concentrations of other
phytohormones (Vreeburg et al., 2005; Pierik et al., 2006).
For example, under phosphate deficiency ethylene promotes
primary root cell elongation but in phosphorus (P)-sufficient conditions it is inhibitory (Ma et al., 2003), suggesting
profound alterations in signalling and regulation of growth
machinery. Ethylene is a positive regulator of [Ca2+]cyt-
dependent root hair elongation. The Arabidopis root hair
apex contains a PM HACC activated by extracellular hydroxyl radicals (another end product of NADPH oxidase
activity, in this case AtrbohC; Foreman et al. 2003), and this
channel may be a downstream ethylene target. The elonga-
tion zone epidermis contains a hydroxyl radical-activated
HACC and a peroxide-activated HACC, which are thought
to operate in growth (increasing [Ca2+]cyt to stimulate
exocytosis) and stress signalling, respectively (Foreman
et al., 2003; Demidchik et al., 2007). We now need more
information on regulation of such channels as a function of
nutrient status to make sense of the biphasic effects of
ethylene on growth. Additionally, as the concentrations of
ethephon used by Zhao et al. to activate HACC induce
cell death in rice (Oryza sativa) epidermis (Steffens & Sauter,
2005) and ethylene potentiates ROS production in cell
death of tomato (Solanum lycopersicum) suspension cells
(Moeder et al., 2002), it would be timely to consider (ROS-
activated) HACC involvement in regulated cell death. As
ABA is a HACC activator via ROS (e.g. Murata et al., 2001)
and can also counteract ethylene-induced death (Steffens &
Sauter, 2005), it may be that fine-tuning of HACC activity
through antagonistic ethylene and ABA signalling pathways
determines whether a cell lives or dies.

Ethylene may also be involved in transcriptional adaptation
to low nutrient availability. Potassium (K)-deprived Arabi-
dopis roots increase transcription of ethylene biosynthesis
genes and ethylene production as part of their adaptive
response in which genes for K+ transporters are up-
regulated (Shin & Schachtman, 2004). This is also tied in
with activity of a specific NADPH oxidase (AtrbohC) and
accumulation of intracellular H2O2 primarily in fully expanded epidermal cells (Shin & Schachtman, 2004). Here
again, we have the possibility of ethylene acting upstream
of an NADPH oxidase, but are there HACCs down the line? The same cells contain a HACC that is activated by
intracellular H2O2 (Demidchik et al., 2007) but also a Ca2+-
permeable nonselective cation channel (Demidchik et al.,
2003) that is activated by extracellular hydroxyl radicals. Both of these channels would be competent to raise [Ca2+]cyt
as an adaptive signal.

Future prospects

The list of HACC activators now includes ethylene. How
and why activation is achieved may be most easily addressed
in a single cell system such as the guard cell or root hair. The
implications of the work by Zhao et al. extend across species
and encompass stress and developmental programming not
only by ethylene but also by other phytohormones. There is
a yawning gap to be filled between the ethylene receptor and
the channel; whether NADPH oxidases are involved is a key
question to be answered. Arguably the most urgent question
is the molecular identities of the HACCs themselves. Only
when the encoding genes are identified can they be placed
with confidence in signalling and regulatory pathways.

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References

Bienert GP, Moller ALB, Kristiansen KA, Schulz A, Moller IM,
Schjoerring JK, Jahn TP. 2007. Specific aquaporins facilitate the
diffusion of hydrogen peroxide across membranes. Journal of Biological
Chemistry 282: 1183–1192.

Free oxygen radicals regulate plasma membrane Ca2+- and K+-permeable

response of Arabidopsis thaliana root epidermal Ca2+ flux and plasma
The ins and outs of stable isotopes in plants

Plant responses to their environment are central to understanding plant function and for assessing their responses to ongoing climatic changes. Metabolite levels, gene activities and metabolic fluxes can be used to study plant responses, but these measures have limitations, for example when it comes to integrating in space and time. This is where stable isotopes come into the picture. Stable (i.e. nonradioactive) heavy isotopes of hydrogen, carbon and oxygen are present in plant substrates and in plant material. Their abundances, which can be measured with very high precision, become modulated both by environmental influences and by physiological and biochemical processes in plants (Fig. 1).

Fig. 1 Isotope signals in plants. The ‘δ value’ is the usual measure for isotope abundance, isotope fractionation processes in the plant are shown in italics, open arrows denote water fluxes with different isotope signatures. The term ‘isotopomer distribution’ reflects the fact that the intramolecular positions of biochemical metabolites contain distinct isotope abundances. In the glucose formula, the variable ‘D’ font sizes illustrate the isotopomer distribution of the hydrogen isotope deuterium.


fractionation processes in the plant. Thus, the isotope abundances of leaf water, primary photosynthates, stored metabolites, and of O$_2$ and CO$_2$ leaving the plant, report on environmental conditions and on the physiological status of the plant. Therefore, stable isotopes have several attractive features. First, modulation of their abundances by plant processes can be related directly to metabolic regulation. Second, they can be used to integrate in space, because they can be sampled from the leaf level to the level of the global atmosphere. Finally, integration in time can be achieved by studying records of stable isotope abundance, for example from tree ring series. Thus, stable isotopes can be used to study plant–climate interactions on vast spatial and temporal scales. However, that isotopes are influenced by environment and by plant physiology is also a curse: to interpret isotope signals, one has to separate both types of influences. In this issue of *New Phytologist*, Gessler *et al.* (pp. 600–613) take a big step forward towards achieving this separation for the oxygen isotope $^{18}$O.

‘... that isotopes are influenced by environment and by plant physiology is also a curse: to interpret isotope signals, one has to separate both types of influences.’

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**Tracking $^{18}$O from leaves to roots**

Stomatal function creates an $^{18}$O signal that might be used as an integrating measure of transpiration and which has been linked to agricultural yield (Barbour *et al.*, 2000). The signal is created by the preferential evaporation of H$_2^{16}$O compared with H$_2^{18}$O, enriching leaf water in H$_2^{18}$O (Fig. 1). Gessler *et al.* ask how this signal is transferred from leaf water to photosynthetic and how it propagates in the plant. The authors’ achievement is to link $^{18}$O in leaf water, in photosynthetic and in phloem, under the dynamic conditions of the diurnal cycles of transpiration and assimilation.

The work combines two methodologies to monitor $^{18}$O variation of different plant parts during the diurnal cycle. First, the $^{18}$O abundance of leaf water is modeled as a function of environmental factors (Farquhar & Cernusak, 2005). Second, the $^{18}$O enrichment of several metabolites with different turnover times is used to track the transfer of isotope signals from leaves down the stem of the plant (Keitel *et al.*, 2003).

Gessler *et al.* calculate the diurnal course of the $^{18}$O enrichment of leaf water using the most current model, which includes $^{18}$O enrichment by transpiration, back-diffusion of $^{18}$O-enriched water against the transpiration stream (the Pélet effect) and hysteresis of the leaf water pool (Fig. 1, insert). The calculated leaf water $^{18}$O enrichment is used to predict the $^{18}$O enrichment of leaf organic matter. The predicted values agree well with the observed values, confirming the model for leaf water $^{18}$O enrichment and its transfer to leaf organic matter. The next step is to convert the diurnal course of the $^{18}$O enrichment – showing a pronounced maximum in the afternoon – into an integrating $^{18}$O signal of water status. To follow the transfer of the $^{18}$O signal in the plant, the authors measured the $^{18}$O abundance of phloem organic matter along the plant axis. This showed that the daily average of the $^{18}$O enrichment is conserved during phloem loading and during transport to the stem base. However, the amplitude of the diurnal $^{18}$O variation is dampened in phloem, compared with leaves, because synthesis and remobilization of transitory starch level out day–night differences of transpiration. Thus, phloem organic matter contains an $^{18}$O signal of the diurnal average of leaf water $^{18}$O enrichment.

The work presented by Gessler *et al.* lays the basis for using $^{18}$O as an integrating measure of the water balance of plants. In addition, the authors were able to draw physiological conclusions directly, in that xylem organic matter has an $^{18}$O enrichment similar to phloem, showing that xylem organic matter originates from xylem/phloem exchange and not from the roots. Finally, the authors raise the important question of whether these results, in *Ricinus*, will apply to other species, especially trees.

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**Deriving multiple signals from stable isotopes**

A mechanistic understanding of $^{18}$O signal transfer in trees would improve tree-ring-based climate reconstruction and studies of plant–climate interactions. In the present study, the modeling of phloem $^{18}$O enrichment relied on the known source water $^{18}$O abundance. In contrast, in applications to tree rings, the source water $^{18}$O abundance is itself an unknown variable containing interesting information, namely the temperature signal present in the $^{18}$O abundance of precipitation (Fig. 1). So, interpreting the $^{18}$O abundance of tree rings is complicated by the mixing of environmental and physiological influences. Two approaches are emerging to solve this complication, so that both environmental and physiological signals might be recovered.

First, measurements of several isotope abundances can be combined. For example, Scheidegger *et al.* (2000) proposed a model that uses $^{13}$C and $^{18}$O abundances to separate signals reflecting humidity and photosynthetic capacity. However, approaches based on combining several isotopes are hampered when $^{18}$O, D and $^{13}$C abundances are correlated. $^{18}$O and D are fractionated in parallel in the hydrological cycle; and in plants, stomatal conductance has a major influence on all three isotopes. This may constitute a limitation of the
approach, because uncorrelated measurables would be preferable for the reconstruction of multiple signals.

Second, isotope abundance can be measured for specific intramolecular positions of a metabolite (isotopomer distribution; see the glucose formula in Fig. 1). For example, it has been shown that the abundance of $^{18}$O and D varies among the intramolecular positions of the glucose units of cellulose (Betson et al., 2006; Sternberg et al., 2006). For $^{13}$C, isotopomer abundance variation is very likely for cellulose, as it has been observed in plant glucose (Schmidt, 2003). Isotopomer variation reflects the fact that each intramolecular position of a metabolite has a distinct biochemical history; therefore, each isotopomer abundance can, in principle, carry an independent signal. For example, the oxygen and hydrogen atoms bound to carbon 2 of glucose exchange strongly with source water during cellulose synthesis (Sternberg et al., 2006; Augusti et al., 2006). Therefore, the respective isotopomer abundances can be expected to be independent of all leaf-level processes and to depend exclusively on source water isotope abundance. This may open a way to reconstruct the source water isotope abundance, allowing temperature reconstruction and resolving the complication of overlapping environmental and physiological influences. With the source water isotope abundance reconstructed, the work of Gessler et al. might then be used to detect a humidity signal. Along this strategy, several signals may be reconstructed using isotopomer abundances in tree rings. This strategy may allow the detection of adaptations of trees to environmental changes on time scales of centuries, a largely unaddressed question in global change research.

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References


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Rhynie chert: a window into a lost world of complex plant–fungus interactions

Using material from a block of 400-million-year-old Rhynie chert about half as big as a brick, Krings et al. (this issue; pp. 648–657) studied fungi populating 250 rhizomes from Nastis aphylla, an early vascular plant. As Taylor et al. (2004) pointed out, most paleobotanists choose to study beautifully preserved plant material. Krings et al. have made one of the first studies where researchers instead examined a large number of creeping, underground rhizomes in various stages of fungus-induced distortion or degradation.

Modern plants distinguish friends from foes and respond differently to each. Krings et al. sought evidence of active plant responses to fungi in the silicified cellular anatomy of colonized N. aphylla rhizomes. Even in the Devonian, early in land plant evolution, N. aphylla cells responded differently to each of three different fungal colonists. Depending on the fungus, cells in the N. aphylla rhizomes increased the thickness of, and pigmentation in, their cell walls, increased their cell size, or encased fungal hyphae in plant cell wall material. All these morphological responses fall within the range of those shown by modern plants to invasion (Beckman, 1980).

‘Some difficult puzzles are posed by this Rhynie material, such as, how were the spores from fungi dispersed when they were packed into host cells?’
Phylogenetic analysis and divergence time estimates

Before returning to Devonian fungus–plant interactions, we will consider which lineages of extant terrestrial organisms were already present in the Devonian, based on fossils and on phylogenetic evidence; our conclusions are summarized in Fig. 1. For the fungi and protist outgroups, we estimated ages using sequences from James et al.’s (2006) multigene, 214-taxon phylogeny and four calibration points. As calibration points for the fungi, we assumed that the common ancestor of the Glomeromycota, the Ascomycota and the Basidiomycota originated after the origin of terrestrial plants, ~500 Ma based on dispersed spores (Gray, 1985). We assumed that the Glomeromycota stem lineage was at least as old as 460 Myr based on Glomeromycota-like spores from the Ordovician (Redecker et al., 2000). We fixed the time of the divergence of monocots from dicot plants at 168 Ma (Magallón & Sanderson, 2005) and that of the first divergences among the multicellular animals at 695 Ma (Douzery et al., 2004).

The James et al. (2006) alignment (available at http://www.aftol.org/alignments/comb_prot_ex.nex) consisted of 2108 amino acid positions from protein-coding genes (elongation factor 1-alpha and the largest and second largest subunits of DNA-dependent RNA polymerase II) and 2244 nucleotide positions (ribosomal DNA large, small, and 5.8S subunits). We reduced James et al.’s (2006) original 214 taxa to 25. To infer a phylogeny with branch lengths, we used MrBayes 3.1.2 (Huelsenbeck et al., 2001), allowing the program to estimate the proportion of invariant sites and the gamma shape parameter separately for the DNA and amino acid regions, and to select an appropriate model of amino acid substitution for the protein portion of the alignment. We used two independent runs of 500 000 generations each, sampling trees every 1000 generations. To estimate posterior probabilities for a consensus topology with branch lengths, we used a set of 500 trees, 250 per run, sampled after 250 000 generations, at which time the log likelihoods for each run had converged and reached a plateau.

We estimated divergence times by the penalized likelihood method with a truncated Newton algorithm, using the computer package r8s, version 1.70 (available from M. Sanderson at http://ginger.ucdavis.edu/r8s/) with the consensus topology and branch lengths from the Bayesian input tree. As an outgroup, we chose Toxoplasma gondii, a taxon that does not appear within the plant, animal and fungal lineages. Following Bayesian analysis, we removed T. gondii from the resulting tree, leaving a tree with 24 taxa, and a basal trichotomy with branch lengths, as required by the r8s computer programs. We tested for possible alternative equally likely estimates for node ages with the ‘Checkgradient’ option of the penalized likelihood program and set the analysis for 10 replicates under different starting conditions, using ‘set num_time_guesses’. Penalized likelihood can compensate for substitution rate variation among lineages if ‘smoothing’ is optimized to take advantage of statistical correlation of rates within lineages. We optimized ‘smoothing’ at 5600 to correct for autocorrelation (r8s, version 1.70, user’s manual, available from M. Sanderson at http://ginger.ucdavis.edu/r8s/). This degree of smoothing was high and it indicated a large amount of substitution rate variation across lineages. Such variation was a reminder that estimates for ages of fungal groups have a large and unknown amount of error resulting from inconsistent substitution rates as well as from possible errors in calibration or in estimation of phylogeny (Taylor & Berbee, in press). The resulting phylogeny (Fig. 1) was consistent with the tree from the complete 214 taxa from James et al. (2006) in showing the Glomeromycota, zygomycetes and Chytridiomycota as paraphyletic to one another, but the Bayesian support for this branching order was not overwhelming. Alternative trees with reasonably high likelihoods showed a monophyletic clade that included the nycoribrial Glomeromycota as well as saprobic species such as Rhizopus oryzae and commensals such as Smittium culisetae, a grouping also found by Liu et al. (2006). Whether these fungi were paraphyletic or monophyletic, phylogenetics and fossil evidence supported their presence in the Devonian, early in the history of vascular plants (Taylor et al., 2004).

Candidate groups of fungi or fungus-like heterotrophs; which were possible relatives of N. aphylla fungi?

With an estimate of fungal divergences in time (Fig. 1), we can see that the extant fungi most similar to the N. aphylla fungi are from basal lineages (Fig. 1). These include zygomycetes, which share aseptate hyphae with some of the fossils, and Chytridiomycota water molds, which, like others of the fossils, have spherical thalli. However, fungi are not the only plant pathogens with a long evolutionary history. Oomycetes and plasmodiophoromycetes are nonfungal protists that were likely present in the Devonian (Fig. 1) and could account for some of the N. aphylla ‘fungal’ remains. The oomycetes originated from a photosynthetic ancestor also common to brown algae and diatoms; oomycetes convergently evolved fungus-like hyphae and an absorptive mode of nutrition. Oomycetes cause disease in many land plants (late blight of potatoes is one such example) and, like the N. aphylla fungi and living zygomycetes, their hyphae lack regular septa. Because of the lack of available sequences, plasmodiophoromycetes were not included in Fig. 1, but they are biotrophic pathogens related to foraminifera (Archibald & Keeling, 2004) that infect a variety of hosts, including oomycete fungi and vascular plants. In plant cells, plasmodiophoromycetes form zoosporangia as well as thick-walled resting spores which are released when the plant roots disintegrate. They, like Chytridiomycota, could be responsible for spherical thalli within plant material.
Comparison of Devonian and modern fungi in plants

A strength of the Krings et al.’s paper is that each of the three fungi was found many times. In the absence of identifying characters, Krings et al. referred to each kind of fossil fungus by number. ‘Notitia aphylla fungus no 1’ consisted of narrow, aseptate hyphae and wider ‘spores’ and, although this was the least frequent of the fungi, it was found in 15% of the axes, or 37 of the 250 examined. The fungus caused damage in plant tissue, judging from the disintegrated host cells lying below the region of cells filled with fungal hyphae and spores. Evidence that the host was alive at the time of infection is provided by a layer of thicker, darkened cell walls that separated the zones with and without fungus no 1 in the infected N. aphylla rhizome. The formation of thickened cell walls and darkening of tissues are common responses of living plants to fungal invaders. Other evidence that the host was alive at the time of fungal colonization is provided by a layer of granular material covering hyphae found in conducting cells and a thickened and twisted rhizoid that was colonized by fungus.

Some difficult puzzles are posed by this Rhynie material, such as, how were the spores from fungi no 1 and no 2 dispersed when they were packed into host cells and, in the case of fungus no 1, attached to hyphae? In modern ecosystems, spores of Ascomycota, Basidiomycota and zygomycetes fungi, and also of oomycetes, form on the outside of their plant substrate, often responding to environmental cues such as light, surface features, and oxygen availability. In these fungi, dispersal is effected by wind, water or animal movement. In the Devonian samples, large numbers of spherical spore-like structures were packed within cells of decaying tissues. Modern fungi immersed in underground plant tissue release motile spores capable of moving through viscous decaying matter, or they release spores that swim away once the plant material has been lost to decay. Could spores in the N. aphylla rhizomes have germinated into crawling plasmodia, as in some stages in the plasmodiophoromycetes? Or, might they have directly released flagellated zoospores like modern oomycetes or Chytridiomycota, or released zoospores after the further disintegration of surrounding tissue, like modern plasmodiophoromycetes?

Comparison with fungi associated with sporophytes of extant plants provides little help in identifying fungi no 1 and no 2. The closest taxonomic relationships of N. aphylla may be with the Zosterophyllophyta (Krings et al., 2007), in which case, its closest living relatives may be lycophytes, or club mosses. Unlike the Devonian fossil fungi, most fungi recorded from modern lycophytes have regularly septate hyphae and are members of Ascomycota and Basidiomycota (Farr et al., 1989). The oomycete reported from lycophyte sporophytes, Phytophthora cinnamomi (Farr et al., 1989), has aseptate hyphae but no other characters in common with the N. aphylla fossils.

However, the gametophytes of several early diverging land plants have mycorrhizal, Glomeromycotan fungi that resemble fungus no 3 in the N. aphylla rhizomes. Glomeromycota has
the best continuous fossil record of any group of fungi, beginning with the 460 Ma Ordovician spores that serve in Fig. 1 as a calibration point marking the minimum age for the Glomeromycota stem lineage (Redecker et al., 2000). By the Devonian, fossil evidence for the Glomeromycota included structures that modern vesicular mycorrhizal fungus form in their hosts, such as arbuscules (the highly branched fungal hyphae within host plant cells that are perhaps the main site of plant–fungal nutritional exchange), vesicles, aseptate hyphae, and spores (Remy et al., 1994). As might be expected if it were a mutualistic mycorrhizal partner, fungus no. 3 was common, colonizing more than 95% of rhizome segments. As in modern fungal partners of lycophytes (Schmid & Oberwinkler, 1993) and other nonflowering plants such as the fern ally Ptilotum nudum (Duckett & Ligrone, 2005) and the liverwort Marchantia (Russell & Bulman, 2005), the hyphae did not produce arbuscules. However, the hyphae of the fungus did produce vesicles or give rise to thick-walled spores.

Somewhat surprisingly, hyphae from fungus no. 3 were encased with what Krings et al. interpreted as plant cell wall material. Encasement is a common response of a plant to invasion of its cells by a plant pathogen, for example, by the haustorium of a rust fungus or powdery mildew. An encasement layer may form around the hypha of a mycorrhizal fungus as it enters a cell, but hyphae of extant vesicular–arbuscular mycorrhizal fungi are not usually surrounded by a regular sheath of plant cell material (Schmid & Oberwinkler, 1993; Duckett & Ligrone, 2005). In extant nonflowering plants, mycorrhizal fungi usually produce coils of thin hyphae within host cells, but hyphal coils were missing from fungus no. 3 in N. aphylla. Possibly, the hyphal coils were missing because the Devonian fungi, although representing Glomeromycota, differed just as much from their closest living relatives as N. aphylla does from any living lycophod.

Not only have plants changed since the Devonian, but fungi must also have changed, and not necessarily in faithful coevolutionary lock step with plants. If perfect coevolution were the rule, then the fungus that was the most recent common ancestor of one Glomeromycota species from a liverwort and a second Glomeromycota species from a flowering plant would date back to the Silurian origins of its host lineages. A sample of fungi from divergent host groups would then serve as a good sample of the fungi originally associated with ancient plants. Unfortunately, however, the Glomus species from the liverwort Marchantia are relatively recent and are nested among Glomus species from flowering plants (Russell & Bulman, 2005). Figure 1 suggests that the first division of the Glomeromycota into surviving lineages may have occurred well after early land plant radiations. Not only the Glomeromycota but also the Ascomycota and Basidiomycota coalesce to one Devonian species each. Although the diversity of Devonian fungi may have been less than what is presently found, it had to be greater than three species. The small sample size of surviving lineages from the larger pool of Devonian fungi limits what phylogenetic inference from living fungi can tell us about the ecological relationships of their ancestors. In light of Devonian diversity that surely has been lost, fungal fossils from Rhynie chert provide a remarkable window, in fact, the only window, into a lost world of complex fungus–plant interactions. Krings et al. have taken a great stride in beginning to interpret the view from that window.

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References


Small RNAs hit the big time

Gene silencing: the biology of small RNAs and the epigenome – 24th Symposium in Plant Biology, Riverside, California, USA, January 2007

Small RNAs have exploded on the scene as ubiquitous, versatile repressors of gene expression in plants, animals and many fungi. These small RNAs (21–26 nt) are cleaved from a double-stranded (ds) RNA and induce effects through homologous sequence interactions. Small RNAs come in various forms: microRNAs (miRNAs), heterochromatizing RNAs, tiny noncoding RNAs, short interfering (si) RNAs, natural antisense short interfering RNAs (nat-siRNAs) and small temporal (st) RNAs. They control mRNA stability or translation, or target epigenetic modifications of DNA or histones at specific regions of the genome. Small RNAs have established a new paradigm for understanding eukaryotic gene regulation. The pivotal discovery of dsRNA and its role in RNA silencing in the cell was acknowledged by the award of the Nobel Prize in Medicine or Physiology in 2006 to Andrew Fire (Stanford University, CA, USA) and Craig Mello (University of Massachusetts, Worcester, MA, USA), 8 yr after the seminal publication (Fire et al., 1998). In January 2007, the University of California at Riverside hosted a meeting to discuss the recent advances in small RNAs and epigenetics within the plant science field. In this article, we present the highlights of that meeting, summarize the current status of the field in plants, and point out areas yet to be explored.

‘… Arabidopsis currently has the largest data set of small RNAs of any species with 300 000 unique small RNAs …’

Small RNAs, the genetic dark matter of the cell

Appreciation of the number and complexity of small RNAs in plants and other organisms has been accelerated by revolutionary advances in high-throughput nanosequencing technology (Fig. 1). Pyrosequencing and sequencing by synthesis methods now allow 500 000 small RNA sequences to be generated in a single experiment (Margulies et al., 2005). This is typified by Arabidopsis, which currently has the largest data set of small RNAs of any species, at 300 000 unique small RNAs and growing (Henderson et al., 2006; Lu et al., 2006; Rajagopalan et al., 2006; Fahlgren et al., 2007). These sequences, along with current small RNA sequencing projects in model organisms and agronomically important species such as Physcomitrella patens, Chlamydomonas reinhardtii, rice (Oryza sativa) and maize (Zea mays), will provide a valuable resource for the scientific community to exploit (Axtell et al., 2006; Johnson et al., 2006; Qi Yijun, National Institute of Biological Sciences, Beijing, China; David Baulcombe, The Sainsbury Laboratory (TSL), John Innes Centre (JIC), Norwich, UK; Pam Green, Delaware Biotechnology Institute (DBI), Newark, DE, USA).
ambitious project underway at the Blake Meyers (DBI, USA) and Pam Green laboratories is to sequence small RNAs from about 30 plant species, a task that will undoubtedly provide a rich resource for the community.

Beyond cataloguing the small RNA populations, researchers are using small RNA sequence data to ask questions about the evolution and biogenesis of small RNAs. Jim Carrington (Oregon State University, Corvallis, OR, USA) presented data to support the hypothesis that several nonconserved miRNAs in Arabidopsis are the evolutionary intermediates between genome rearrangements and a miRNA (Fahlgren et al., 2007). This work highlights the power of bioinformatic analysis of large data sets.

**Small RNA biogenesis and mechanisms of action**

The sequencing of small RNAs (sRNAs) from Arabidopsis has led to the identification of abundant 21- and 24-nt RNAs. Twenty-one-nucleotide RNAs target cleavage of mRNAs and guide the cleavage and subsequent phased production of transacting siRNAs (tasiRNAs). The production of 21-nt small RNAs has also been linked to cell-to-cell signalling, although longer dsRNA movement cannot be ruled out (Dunoyer et al., 2005). Twenty-four-nucleotide RNAs are required for RNA-directed DNA methylation and heterochromatinization in Arabidopsis (Qi et al., 2006); however, the exact mechanism of this is still to be elucidated. Large-scale sequencing of heterochromatic small RNAs and the genome-wide description of DNA methylation in Arabidopsis suggest where the 24-nt RNAs may function; however, whether the sRNAs are a cause or a consequence of DNA methylation is still to be determined (Zhang et al., 2006; Steve Jacobsen, University of California, Los Angeles, CA, USA).

An open question is whether 21-nt, 24-nt or longer RNAs are involved in long-distance spreading of silencing. Elegant grafting experiments in a transgenic Arabidopsis system hint at longer dsRNA as a phloem-transmitted signal (Bernard Carroll, University of Queensland, Brisbane, Australia). In this model, the transported dsRNA is then incorporated in cells that receive the signal into a 24-nt small RNA generating pathway for RNA silencing.

Small RNAs (21–24 nt) are presumed to be generated through Dicer cleavage of a long dsRNA intermediate. Recent work has teased apart the overlapping functions of the four Arabidopsis Dicer-like proteins (Herve Vaucheret, INRA, Versailles, France; Olivier Voinnet, Institut de Biologie Moléculaire des Plantes, Strasbourg, France). Intriguingly, recent published and unpublished data sets have also identified larger (26, 28 and > 30 nt) small RNAs (Katiyar-Agarwal et al., 2006; Hailing Jin, University of California, Riverside, CA, USA). How are these larger small RNAs generated? One hypothesis is that they are not generated through Dicer cleavage, but rather through the interrupted action of an RNA-dependent RNA polymerase, as has been shown in *Caenorhabditis elegans* (Pak & Fire, 2006; Sijen et al., 2006).

**Beyond small RNA sequencing**

The incredible growth of small RNA data presents several challenges for the near future. Technological advances have allowed massive data sets to be generated, but there is an immediate requirement for a central database for depositing small RNA sequences. There is also a need for generation and sharing of bioinformatic tools for analysis. Bioinformatics will be integral to readily manipulating large data sets, allowing cross-species comparisons, and assaying genome-wide changes in small RNAs.

Another challenge of the field is a detailed description of small RNAs at the organ and ultimately cellular levels, as well as elucidation of how small RNAs respond to environmental changes. A step towards these goals is the deep sequencing of small RNAs from heat- and salt-stressed Arabidopsis by the Meyers and Green laboratories. Another interesting area is natural variation in methylation patterns. Eric Richards (Washington State University, St Louis, WA, USA) has taken the approach to look for natural variation in methylation patterns in Arabidopsis and to uncover genetic elements involved in this process.

Arabidopsis contains four Dicer-like proteins, six RNA-dependent RNA polymerases, and 10 Argonaute proteins. This diversity allows for complexity in small RNA biogenesis, and emerging data suggest that these proteins can act as modules to be interchanged to generate functional diversity. An example of modularity can be found in the newly identified miRNAs that are Dicer-like (DCL)4 dependent rather than DCL1 dependent (Rajagopalan et al., 2006) or in a transgene silencing system in which dsRNA production by Polymerase (Pol)IV is uncoupled from downstream processing by DCL3 and Argonaute (AGO)4, as with endogenous siRNAs, and instead is linked to DCL4 and AGO2 (David Baulcombe, TSL, JIC, Norwich, UK). These findings challenge our current view of discrete, linear small RNA pathways.

Histone modifications have also been linked to small RNAs in plants (Onodera et al., 2005; Huettel et al., 2006). The quest for a full description of histone modifications – how they are added and removed, and exactly how they impact on gene regulation – is an emerging area. Steve Jacobsen (University of California, Los Angeles, CA, USA) has followed on from his genomic DNA methylation work to describe genome-wide histone methylation in Arabidopsis. He has shown that H3K27 trimethylation is generally present in small domains and is anticorrelated with small RNA accumulation. More detailed analysis of small RNA-directed histone modification was presented by researchers of silencing in *Schizosaccharomyces pombe* (Danesh Moazed,
Harvard University, Boston, MA, USA; Robert Martienssen, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA).

Perhaps the most important challenge facing the small RNA community is linking cellular small RNAs to biological function. Small RNAs have been implicated in viral defence for several years. Recent work has expanded the role of sRNAs into defence against bacterial pathogens, in particular how pathogen miRNAs and host miRNAs contribute to pathogenesis and defence (Katiyar-agarwal et al., 2006; Navarro et al., 2006). Small RNAs also play a role in response to abiotic stresses. Overlapping, convergent transcripts have the ability to form dsRNA and to be processed into siRNAs, leading to regulation of one of the transcripts (Borsani et al., 2005). This has been demonstrated to be important for salt tolerance in Arabidopsis. Small RNA pathways may also be implicated in control of flowering (Caroline Dean, JIC, Norwich, UK).

Future

Further analysis of the abundant small RNA sequence data will undoubtedly lead to an increased understanding of the complex interactions of small RNA-generating loci. We are only starting to appreciate the role of small RNAs in the cell and many questions still exist. How dynamic are these small RNA populations? What are the diurnal changes, and many questions still exist. How dynamic are these only starting to appreciate the role of small RNAs in the cell complex interactions of small RNA-generating loci. We are will undoubtedly lead to an increased understanding of the

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References


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