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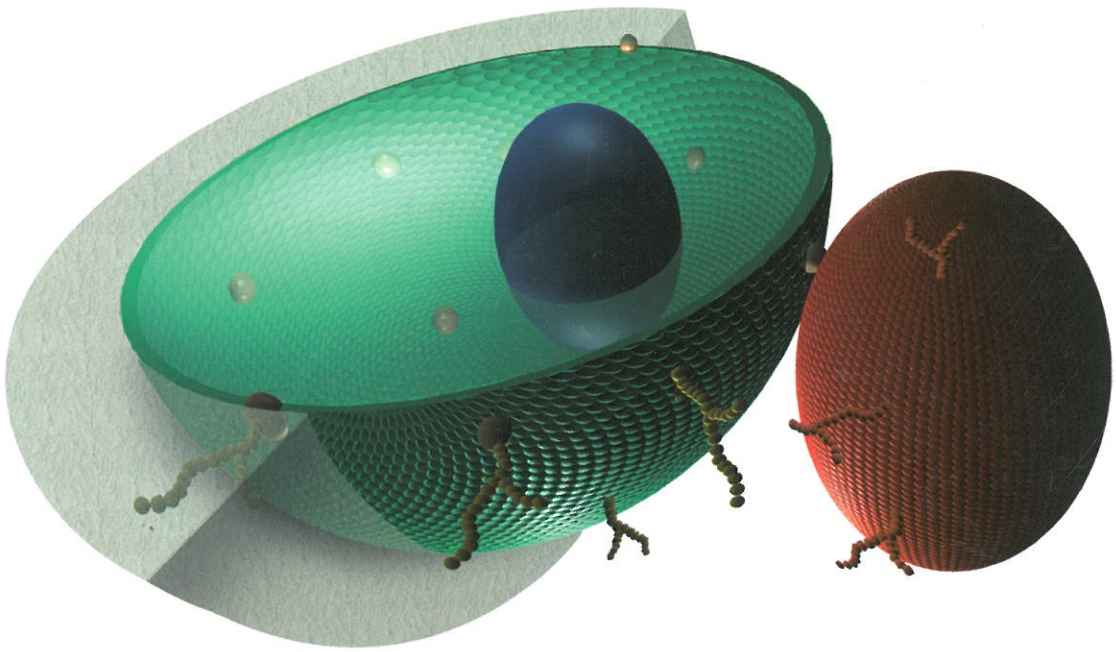

The Interactive Cell

Inside

The Research School of Biological Sciences is one of Australia's leading centres for basic biological research and graduate training. Since its inception in 1967, it has focussed on three domains: plant science, genetics, and neuroscience. This work is carried out in 11 research groups, organised as follows:

Visual Sciences aims to better understand how the optical image captured by the eye is analysed by the visual system to produce perception. To maximise the chances of success in this enterprise, research has concentrated on simple visual systems such as those of insects. Group leader: Dr Srini Srinivasan.

Plant Cell Biology aims to explain the cellular basis of plant development and how it is regulated. The group also investigates cell division in plants using the latest techniques of molecular biology and molecular genetics. Group leader: Professor Brian Gunning.

Molecular and Population Genetics examines biological problems with genetic and molecular techniques using two organisms — yeast and the fruit fly (*Drosophila*). Because yeasts can be grown and manipulated like bacteria, they are ideal model organisms for experiments in cell biology.
The research on *Drosophila* concerns the micro-evolutionary features that affect genetic variation in natural populations.
Group leader: Professor John Gibson.

Plant Microbe Interaction studies plant pathology, resistance, symbiosis and defence systems.
A particular focus of the group is the engineering of nitrogen-fixing bacteria for more effective nodulation of legumes. Group leader: Professor Barry Rolfe.

Molecular Evolution and Systematics applies the latest techniques in molecular and information science to study the evolution, systematics, identification and functional biology of selected organisms important to Australia. The work includes study of viruses, their vectors and hosts, as well as the comparative molecular biology of development.
Group leader: Professor Adrian Gibbs.

Ecosystem Dynamics seeks to understand the structure and function of plant and animal communities. It develops theories of complex community dynamics, and how communities respond to environmental variations. The aim is to formulate models that can predict how communities will respond to different environments. Group leader: Dr Ian Noble.

Plant Molecular Physiology conducts research into how physiological, biochemical and molecular attributes of individual plants contribute to their fitness in agricultural and natural ecosystems. A long-term goal is the identification of genetic elements that could be incorporated into commercially important agricultural species. Group leader: Dr John Andrews.

Environmental Biology conducts research into how ecological, physiological and biochemical attributes of individual plants contribute to their fitness in agriculture and natural ecosystems. A key theme is photosynthetic carbon gain in relation to growth, respiration, decomposition and water use, all in the general context of global change.
Group leader: Professor Graham Farquhar.

Eukaryote Chromosome Organization studies the biophysical and molecular structure of the chromosome and its role in the evolutionary process. The group uses Australian fauna and flora to investigate the function of chromosomal variation in adaptive evolution and the origin of new species.
Group leader: Dr David Shaw.

Director's Research Unit researches fundamental aspects of photosynthesis using novel techniques in biophysical chemistry, biochemistry and cell physiology, and explores their application to eco-physiological problems. Understanding photoinhibition — the process that reduces the efficiency of photosynthesis at high light levels — is pivotal in learning how plants respond to environmental and biological stresses.
Group leader: Professor Barry Osmond.

Developmental Neurobiology studies the development and activity of nervous systems. Most work involves the sensory systems of hearing, vision and touch, using the sensors of mammals, birds and crabs, insects and spiders. Much of the research makes use of an extensive wallaby colony, a facility that allows study of the coordinated development of vision and hearing in pouch young.
Group leader: Professor Richard Mark.

If you would like to know more about any of the research activities at RSBS, you are welcome to contact the principal researchers involved. The address is: Research School of Biological Sciences, GPO Box 475, Canberra, ACT 2601. Phone: (06) 249 2999 FAX: (06) 249 4891

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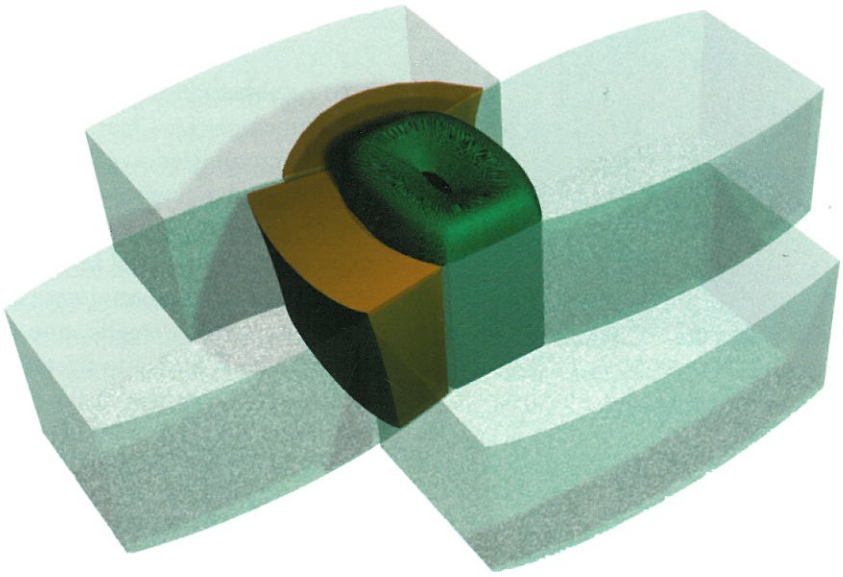
Actin in action



Actin is a protein found in plant and animal cells. In plants it is a major component of the cell 'skeleton' or cytoskeleton.

By developing resourceful and novel experimental techniques for use in combination with new technology, Dr Ann Cleary has cast actin in a leading role in the action of cell division. Her basic research questions how an actively dividing cell 'knows' the location of the division site or, in other words, exactly where to split into two daughter cells. She has discovered that there is an absence of actin at the cell's division site — creating an 'actin-depleted zone', which may be crucial for cell division.

Unlike animal cells, plants have cell walls. These are rigid structures so, once formed, plant cells are unable to change position or dramatically change their shape. Thus, in plants it is the cytoskeleton of individual cells which ultimately governs the development of the plant's shape (morphogenesis).

Most plant cells divide symmetrically during cell division (mitosis). Yet there are some plant structures which require asymmetrical cell divisions to generate their shape. Dr Cleary has used the stomatal complexes of Tradescantia (a herbaceous, perennial plant) as a model for examining asymmetrical cell division and in particular, the role of actin during this process.

Stomata are pores in the leaves of plants which facilitate gas exchange between the leaf and the environment, which is required for photosynthesis and respiration. The development of a stomatal complex requires precise regulation of a sequence of symmetrical and asymmetrical cell divisions. Briefly, a specialised cell called the guard mother cell (GMC), divides symmetrically (longitudinally) to produce the guard cells that regulate gas exchange. In coordination, four adjacent subsidiary mother cells (SMC) divide asymmetrically to produce subsidiary cells which play a role in controlling opening of the pore for gas exchange (see Figure 1). The two side subsidiary cells have an assymetrical shape like a kidney bean or lens and are, therefore, of particular interest to Dr Cleary.

The cytoskeleton is a structural framework that exists throughout the cell. It consists principally of proteins such as actin and tubulin. For these proteins to provide structural support for the cell they must 'polymerise' or bind together to form long fibre or cable–like structures called microfilaments and microtubules (respectively). Dr Cleary's work focuses on the portion of the cytoskeleton inside the plasma membrane called the cortical cytoskeleton.

Figure 1 (above). The development of the stomatal complex from a sequence of symmetrical and asymmetrical cell divisions. Adjacent subsidiary mother cells (light green) divide assymetrically to produce subsidiary cells (orange) that aide in gas exchange through pores in the plant's leaves.

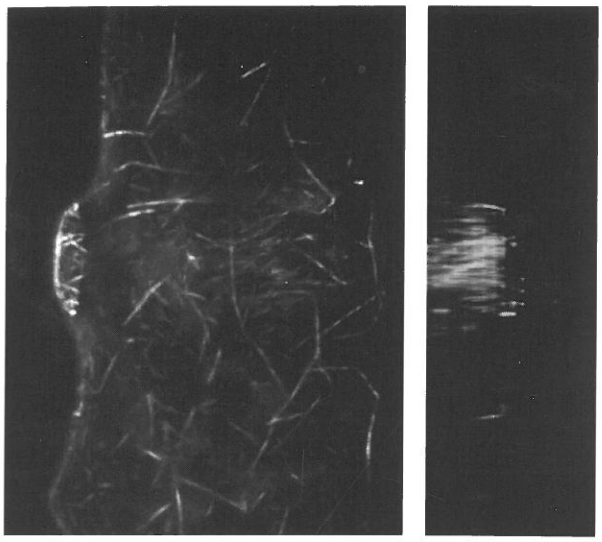


Figure 2. The build up of actin between the subsidiary mother cell's (SMC) nucleus and the adjacent guard mother cell (GMC).

Researchers have previously shown that bands of parallel microfilaments and microtubules, found in the cell cortex, predicted the site of cell division early in mitosis. These studies looked at microtubules and actin arrays in fixed (formalin preserved) cells. During the fixation procedure it is possible that the fine cortical cytoskeleton may have incurred some damage. For this reason Dr Cleary designed a method for examining cell division in living stomatal complexes.

An 'epidermal peel' is an old technique used by Dr Cleary to procuret of live stomatal complexes and is similar to peeling skin from a tomato. Peels are made from the young leaves of a *Tradescantia virginiana* shoot and placed, cuticle side up, in a well slide. The cells in the peel will remain viable for several hours and the developmental changes in the organisation of actin during cell division may be monitored.

To be able to visualise actin in the living stomatal complexes Dr Cleary developed a procedure for the microinjection of individual SMCs with a fluorescent dye that detects actin (in both living and fixed cells). The power of this technique is maximised by confocal laser scanning microscopy (see page 9). This special type of microscope can pick up extremely small amounts of dye in an individual cell with a low level of irradiance. The laser detection system in the confocal microscope facilitates 'optical sectioning' through an individual cell, ie. it looks at sections (1 micron thick) through the depth of a cell. These are recorded until the layers of the entire cell have been examined. This information is analysed and reconstructed by computer to produce three dimensional pictures of a SMC's fluorescing bands of actin. These three-dimensional images are further manipulated by computer to animate the images, for example an individual three-dimensional SMC can be rotated in any direction, in order to see the actin from all angles.

Dr Cleary used confocal video microscopy to record actin 'in action' during the mitotic division of SMC cells. Here, individual SMC cells in epidermal peels were injected with dye, and the dynamics of actin in the cell were recorded on video (two dimensional). In this way she has been able to watch cytoplasmic changes, in concert with the changes to actin, in actively dividing stomatal cells.

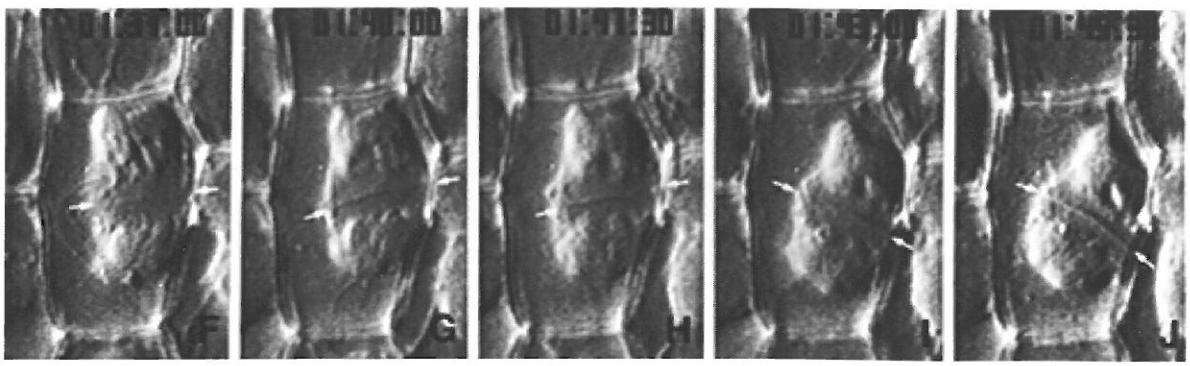


Figure 4. A videoed sequence showing a SMC new cell wall initially being formed above the 'actin depleted zone' and subsequent downward move, so that the site of cell wall formation falls into the actin-depleted zone, allowing the characteristic kidney bean shape of the subsidiary cell to form.

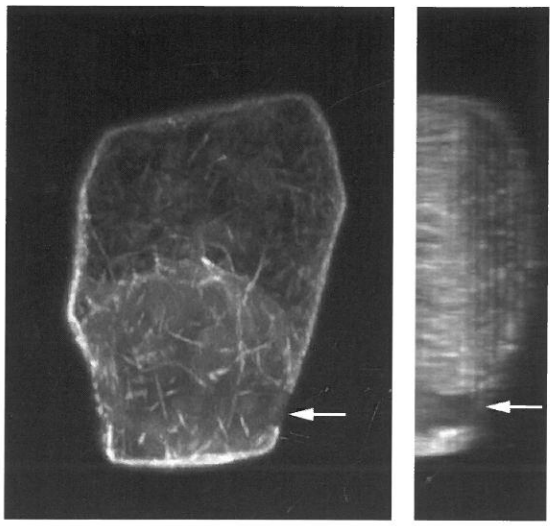


Figure 3. The actin depleted zone.

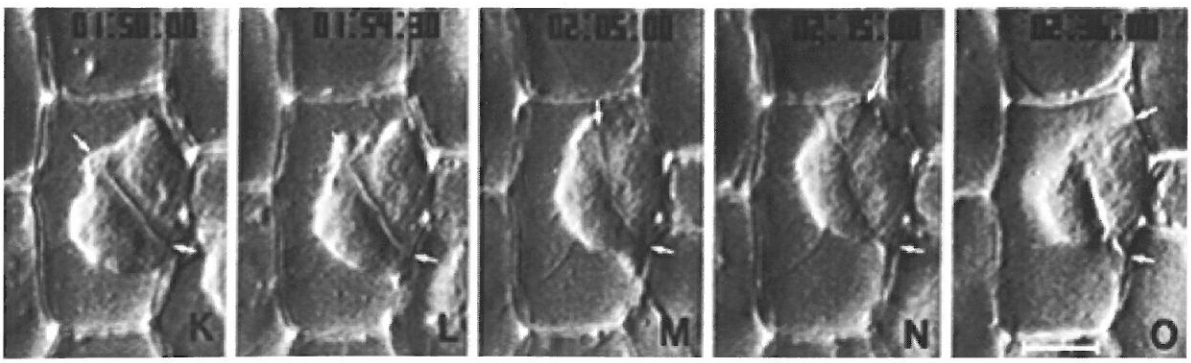
Dr Cleary's innovative experimental design rewarded her by providing new insight into actin's role in cell division. She observed actin in the cell cortex of Tradescantia leaf epidermis cells throughout mitosis. Furthermore, she saw that actin forms many dynamic and complex arrangements during the cell cycle. 'A simplistic analogy for describing the patterns and possibly the role of actin, is to liken it to a road map of cell division,' says Dr Cleary.

During the resting stage between cell division (interphase), cortical actin is present in fine, often transversely oriented filaments. In preparation for a SMC's asymmetrical cell division (preprophase), the nucleus migrates and establishes a polar axis in the cell. Once the nucleus is positioned, thick actin cables build up along the anticlinal (side) wall between the SMC's nucleus and the adjacent GMC (see Figure 2). These persist for the duration of mitosis and their role may be to stablise and secure the nucleus.

Just before the cell divides (late prophase), a subset of cortical actin forms a band which accurately delineates the future site of insertion of the new cell wall. SMCs divide asymmetrically, thus, in these cells this band is contorted in the characteristic 'kidney bean shape' of the subsidiary cell. The most exciting and important discovery was that this actin band disassembles, following the breakdown of the nuclear envelope, creating the 'actin-depleted zone' (see Figure 3). Elsewhere in the cell actin is retained.

The specific disassembly of actin at the cell division site and its maintenance elsewhere during mitosis, results in the division site being enduringly marked by the localised absence of actin. Dr Cleary was the first to describe the 'actin-depleted zone' in living cells.

The video sequence shown in Figure 4 (below) further supports Dr Cleary's claim that actin, or in this case its absence, is a 'road map' showing where the cell will divide. Here, frames F—H show that the cell wall appears to be forming above the actin depleted zone. Frames I—K show that the site of cell wall formation jiggled about and eventually moved down, so that it was finally located in the actin-depleted zone. Frames L—O show that, once the new cell wall was correctly positioned, the characteristic kidney bean shape of the subsidiary cell was formed.



Visiting German Scholar

Ulrike Mathesius is a determined and motivated young scientist, who visited the RSBS for nine months on a student scholarship from the German National Scholarship Foundation (Studienstiftung des Deutschen Volkes). To be eligible for this prestigious award, candidates must rank in the top 1% of German students, then be nominated by an academic for selection and, finally, perform successfully at interview. When considering the high standard set by this selection process, it is not surprising that Ulrike worked successfully in three different research groups during her visit to RSBS; Plant Cell Biology, Plant Microbe Interactions and Molecular and Population Genetics.

Ulrike claims that she has always wanted to be a scientist, definitely since fifth grade at primary school! Ulrike explained that her constant motivation for science comes from the fun and excitement that she experiences while doing science. After finishing school, she spent two years at university (Technische Hochschule Darmstadt), completing her Vor Diplom — a basic science undergraduate degree. Her visit this year to RSBS was her first experience working in a research school.

The role of actin filaments during stomatal cell division (see Actin in Action), was one of the projects Ulrike worked on, under the supervision of Dr Ann Cleary, in the Plant Cell Biology Group. It was Ulrike's task to discover whether actin is broken down during the fixation procedure and therefore, isn't seen in subsequent staining.

Ulrike's research showed that actin is indeed lost or broken down during the fixing procedure. These results will contribute to a joint publication entitled 'Confocal microscopy of F-actin in fixed Tradescantia leaf epidermal and stomatal cells'.

Having completed her nine months with the RSBS, Ulrike has returned to Germany to complete her science studies. She is planning to return here, and who would not believe her when she said of her visit here, 'I am so glad I'm away, completely independent, which means that I can do science all day—it is the best thing in the world!'

These results provide evidence that cortical actin does indeed delineate the cell division site and thus plays the lead role during mitosis. Dr Cleary has also shown that actin-depleted zones are present irrespective of whether the division site is symmetrical (GMC, epidermal cells, subsidiary cells) or asymmetrical (SMC).

Other labs, who use the more traditional methods of cell fixation, have confirmed the presence of cortical actin as well as the strands of actin associated with polarised nuclei. They have not, however, located an actin band or actin-depleted zone delineating the plane of cell division and thus, Dr Cleary's results have generated some discrepancy in the literature.

Ms Ulrike Mathesius, a visiting scholar (see side panel), repeated the labelling of actin, but in fixed Tradescantia cells, while working in Dr Cleary's laboratory. Ms Mathesius' results confirmed those of other research groups — she could not identify actin in bands delineating the plane of cell division in stomatal complexes which had been preserved. Unlike the other researchers, she did however, see actin-depleted zones.

In future experiments, Dr Cleary plans to treat live Tradescantia cells with cytoskeletal and metabolic inhibitors, in an attempt to further elucidate the role of actin in the control of the division site.

Dr Cleary' s basic research has ultimately produced valuable insight into plant morphogenesis. She has, by developing ingenious experimental techniques, shown that actin indeed provides the action required for the precise positioning of a new cell wall during cell division.

If you want to know more

Dr Anne Cleary.

- Microtubule and F-actin dynamics at the division site in living Tradescantia stamen hair cells. A. Cleary, B. Gunning, G. Wasteneys and P. Hepler. Journal of Cell Science (1992), **103**, 977–988.
- F-actin redistributions at the division site in living Tradescantia stomatal complexes as revealed by microinjection of rhodamine-phalloidin. A. L. Cleary. Protoplasma (1995), in press.

- You are welcome to contact Dr Cleary via the address and phone numbers on page 2.

The dynamic structure and structural dynamics of

Microtubules

Microtubules, polymers of tubulin, exist in two 'states' within a cell; one highly dynamic state and one comparatively stable state. Intuitively, it would seem that the more important functional role of microtubules would coincide with an active, rather than stable, state. The structural dynamics of microtubules and corresponding function is, however, not so predictable, as microtubules' major functional role appears to correspond with the stable state. This is just one of the unusual and surprising features of microtubules.

Dr Geoffrey Wasteneys, of the Research School of Biological Sciences (RSBS), is investigating microtubule dynamics in plants. He describes his approach to research as a 'behaviouralist at the molecular level' (rather than at the level of the whole organism), since he observes the 'behaviour' of microtubules in living cells.

Over the past thirty years scientists have been examining microtubules in preserved (fixed) cells. The latter approach has two obvious disadvantages. First, fixed material only provides a glimpse of what is happening at the single point in time when the tissue was fixed. Second, fixatives generally disrupt the native arrangement of microtubules. By observing the dynamics of microtubules in living cells, Dr Wasteneys hopes to understand how these dynamics affect microtubule function as well as gain some insight into microtubules' regulatory processes.

Microtubules

Tubulin is a major structural protein of all eukaryotic cells (those which have a nucleus). Dimers of α and β tubulin link together to form long, stable polymers called microtubules (see Figure 1). In vivo, microtubule assembly requires the presence of microtubule associated proteins (MAPs) and the assembly-disassembly process is controlled by the hydrolysis of guanosine triphosphate (GTP), which binds to tubulin dimers along with the cofactor Mg^{2+}. It is interesting to note that the two ends of a microtubule have different affinity for tubulin dimers. Affinity for dimers is greater at the positive end than at the negative end. When microtubules are in equilibrium with tubulin, dimers may be released predominantly from the negative end and added predominantly to the positive end, a phenomenon known as treadmilling. In this situation the net addition at the positive end equals the net loss from the negative end and there is no polymer growth. This has important implications for stability of microtubules in the cell. Since the concentration of free tubulin in the cell is low, free microtubules would be expected to rapidly depolymerise. For microtubules to form stable structural frameworks (such as those in the cell cortex), their negative end has to be protected from depolymerisation. Generally, microtubules radiate from special regions in cells, called microtubule organising centres, which act to protect and stabilise their negative ends.

It is believed that microtubules exhibit two types of structural dynamics in the cell. 'Treadmilling', is one and 'dynamic instability', which describes the turnover of different sub-populations of microtubules, is the other. In dynamic instability one population of microtubules may be growing slowly, while concurrently a different population will be shrinking rapidly.

The ability of microtubules to exist in a number of dynamic 'states' provides them with the structural flexibility required to serve a myriad of different functions. In animal cells, microtubules form structural frameworks, radiating throughout cells from a centrally located structure, called the

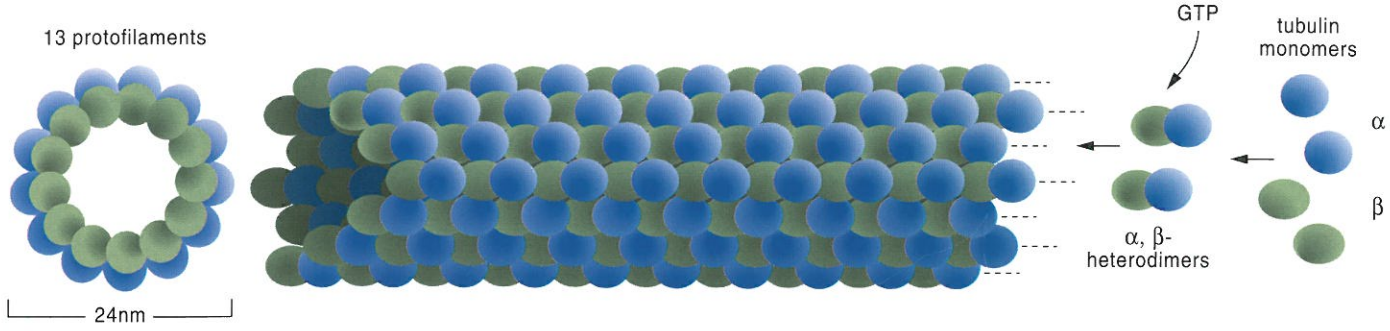


Figure 1. Schematic representation of a microtubule.

Finding out: Confocal Microscopy

In science as in any other walk of life, seeing is believing. Those who study the cell surface need to be able to see it. One new form of microscopy, confocal laser scanning microscopy (CLSM), has proved to be very useful for this purpose. The articles Actin in action and The dynamic structure and structural dynamics of microtubules, illustrate the utility of this powerful new technology.

The idea of confocal microscopy is far from new. The method was patented as long ago as 1957. Impracticable then, it had to await the development of lasers, computer control systems for manipulating the moving parts of the microscope, and software for image processing. All of these components are now available and over the past five years or so confocal microscopes have become indispensable tools for cell biologists. Many thousands have been installed in research laboratories worldwide despite their great expense. There are now more than 30 in Australia.

The principle is simple, the microscope scans and records images of naturally fluorescent or specifically stained components in the specimen being examined (eg. Figures 2 and 3b). A laser beam (pink in diagram below) is focused to a very fine point in the specimen, which responds by emitting fluorescence (green). The 'confocal effect' is provided by an equally fine aperture which selects only the 'in-focus' fluorescence and rejects almost all of the fluorescence from 'out-of-focus' parts of the specimen (gold and yellow). The result is an exceptionally sharp picture of the point in the specimen that is being illuminated by the laser. The image is undegraded by flare from the neighbouring parts of the sample. The laser is scanned over the specimen in the manner of a television raster and the image of the whole plane of focus is built up point by point at high speed by collecting and measuring the fluorescence with a photomultiplier mounted behind the detector aperture. The image is stored in digital form in computer memory.

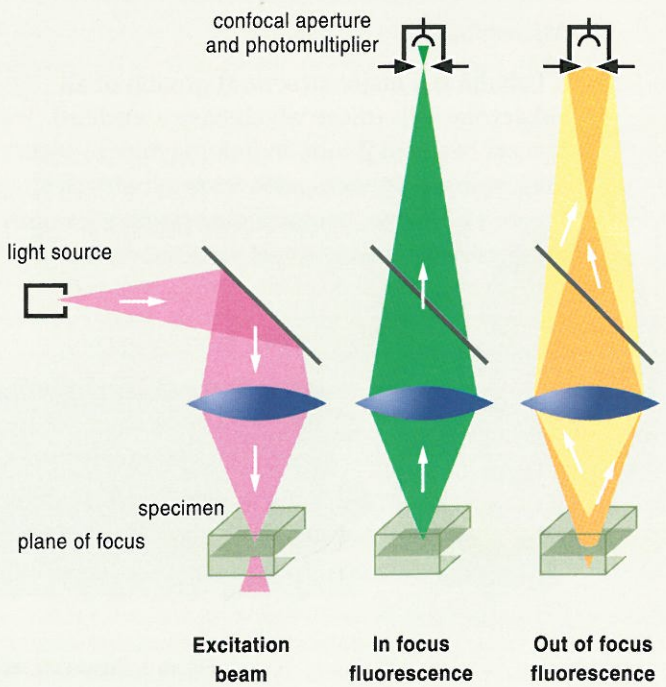
The resolution of the image in the plane of focus is slightly better than in a conventional fluorescence microscope. Where the confocal system excels, however, is in its ability to give clear images of successive levels in the specimen, arising from the way it discards the out-of-focus flare. Recording many images, slightly re-focusing the microscope each time, gives a three-dimensional set of picture points. The specimen can then be examined by computer manipulation of the data points. It can be viewed from any desired direction, or in thick or thin slices, or in stereo views, or in tilting, rotating animations. In combination with image-processing software, objects as small as single receptor molecules on the cell surface can be labelled and made visible to our eyes, enabling us to 'see and believe'.

centrosome. In plant cells, which lack centrosomes, microtubules are, nevertheless, active in the formation and functioning of many cytoskeletal arrays, including mitotic arrays and interphase arrays, involved in cell division. Cortical arrays, involved in changes in cell shape, are of particular interest to Dr Wasteneys.

Microtubules in the plant cell cortex

The relationship between microtubules and cell growth is the same in all plants from many simple green algae through to higher plants. The scientists Ledbetter and Porter found the first evidence of cortical microtubules being important components of cell morphogenesis (see Cellulose the most abundant and elusive biomolecule page 11). Cortical microtubules control the orientation of cellulose during the development of the primary cell wall. Precise rearrangements of cortical microtubules precede any alteration in the direction of cell growth. When a cell stops growing, cortical microtubules become randomised in orientation and depleted in number. One hypothesis suggests that microtubules act as physical boundaries to guide the cellulose synthase enzyme complex as it moves around the cell cortex, extruding microfibrils into the primary cell wall. Scientists are, however, still to determine what regulates the initial orientation of microtubules in the cell cortex!

The principle elements of confocal microscopy



Prior to Dr Wasteneys' research, a great deal of contention existed within the scientific community, in regard to how cortical microtubules were organised. Two major theories, which were dependant upon the method of fixation used, had developed. When plant tissue sections were fixed in formaldehyde, the microtubules appeared to be very closely annealed to each other and seemed to be organised in long interlinked helices. When plant tissue sections were fixed in glutaraldehyde there was a marked difference in microtubule organisation. Here, they appeared to be relatively short independent structures and the microtubules were described as discrete elements. These two theories coexisted and were hotly debated for a number of years. It was not possible to effectively test the validity of these theories until advances in microscopy and immunolabelling provided the tools to look at the arrangement of microtubules in living cells.

Microtubules live

With breakthroughs in techniques and instrumentation in hand, Dr Wasteneys only had to develop a suitable assay for immunolabelling and microinjection of tubulin.

Tubulin is highly conserved across both plant and animal kingdoms. Thus, Dr Wasteneys was able to use tubulin isolated from sheep brains for his microinjection experiments in plants! It took Dr Wasteneys 6 months, and many trips to the abattoir to develop a suitable method to produce a fluorescently labelled and active tubulin probe for his studies.

The fluorescent brain tubulin was microinjected into living cells from the green alga *Nitella pseudoflabellata* and the higher plant *Tradescantia virginiana*. Dr Wasteneys explains, 'as I had been ambitious about the new approach, I had gone in thinking that I might not see anything but I was pleasantly surprised by the beautiful images of individual microtubules' (see Figure 2).

Showing the incorporation of exogenous brain tubulin by cortical microtubules of living plant cells was a world first. More importantly, the arrangement and the dynamic behaviour of microtubules could be observed over a period of hours, using time lapse photography. The results showed that the arrangement of cortical microtubules was similar to that described using glutaraldehyde fixation. To investigate the

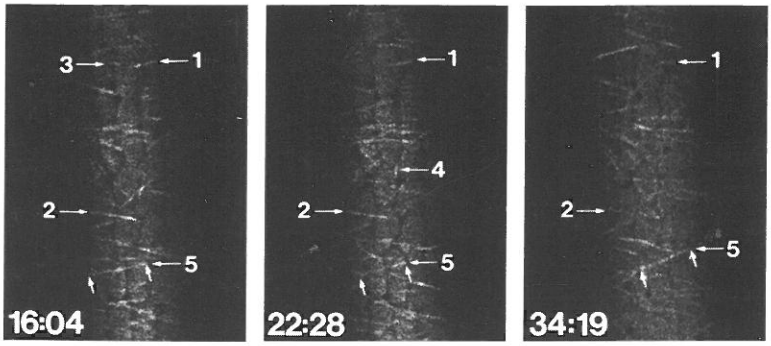


Figure 2. Incorporation of fluorescent brain tubulin into cortical microtubules of Nitella cells.

dynamics of microtubules, different concentrations of the fluorescent tubulin were compared, to ensure that the injection of tubulin had not shifted the dynamics of tubulin in favour of microtubule assembly. As fluorescent tubulin was continually utilised as microtubules were being assembled and disassembled in the cortex, it was assumed that the normal dynamic behaviour of cortical microtubules was being displayed.

'That was absolutely fantastic,' reflects Dr Wasteneys, 'one night gave me the same amount of information as 6-8 months work with old techniques!'

Using 5 to 10 minute intervals to examine changes in microtubule dynamics, showed considerable variation in the life span of microtubules. Some were observed at one time point only, while others remained stable for over 30 minutes, which is a very long time in terms of cellular activity. When the time interval was reduced to 1 minute, it became apparent that the majority of microtubules are labile and transient. These labile microtubules continually ran through assembly/disassembly cycles, while only a small number were more stable, existing for more than 5 minutes. These results clearly show that cortical microtubules are much more dynamic than was previously believed.

In an earlier study, Dr Wasteneys outlined two models to explain how different stimuli may change the direction of cell growth by influencing microtubule orientation. In the first model, 'differential turnover' or 'selective stabilisation', microtubules are relocated by disassembly and reassembly. In the second, 'reorientation model', microtubules remain stable and pivot into a new position. As Dr Wasteneys' results show cortical microtubules to be dynamic, he postulates that changes in the orientation of microtubules is probably accomplished by assembly/disassembly and possibly translocation of microtubules over time periods as short as a minute.

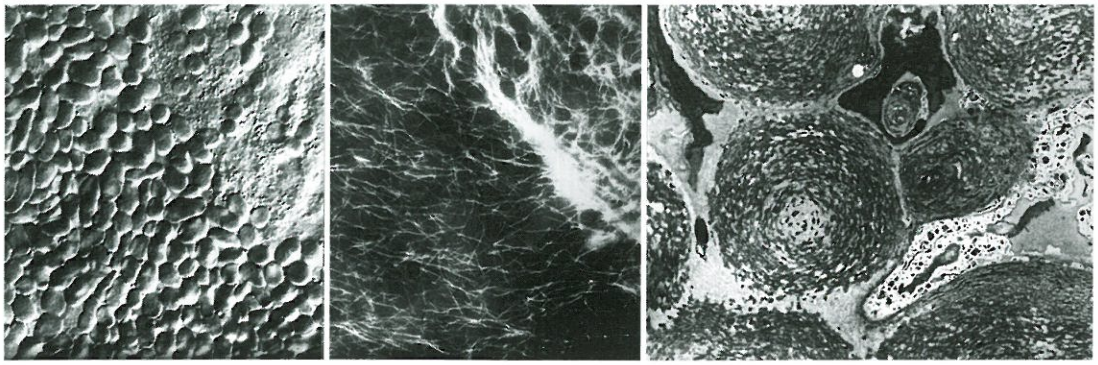


Figure 3. Microtubule orientation in the wound site of a Nitella cell. (a) Chloroplasts are absent from the wound site–upper right hand corner, (b) immunofluorescence shows a distinct change in the organisation of microtubules, from transverse in the unperturbed region to randomised in the wound site, (c) the swirl-like pattern of cellulose at the wound site of a Nitella cell.

Are microtubules involved in wound repair?

In giant cells of the alga Nitella, wounding dramatically alters the deposition of cellulose from the usual transverse alignment into complex spiral patterns (see Figure 3c). It was thought that the reorganisation of microtubules was necessary to coordinate this cellulosic pattern.

Professor Ilse Foissner (University of Salzburg), who first described wounding in Nitella, recently collaborated with Dr Wasteneys to determine the involvement of microtubules (see Figure 3a, b, c). Surprisingly, they found that although microtubules reorganise at wound sites, they play no significant functional role in the process; identical spiral patterns of cellulose occur whether or not microtubules are present.

Drs Wasteneys and Foissner were intrigued with this unexpected finding and went on to compare the dynamic properties of microtubules at undisturbed regions and at wound sites, within single live cells. They discovered that 'functional' microtubules of unwounded regions are, on average, significantly more stable than their disorganised counterparts at wound sites. Thus, it does indeed appear that 'microtubules' major functional role corresponds with the stable state,' rather than with the dynamic state in wound healing.

What is in control of microtubules?

Any model describing regulation of microtubule orientation and cellulose deposition must take into consideration the dynamics of cortical microtubules. Almost nothing is known of MAPs in higher plants but these molecules are thought to play a significant role in the regulation of microtubule dynamics.

Experiments in animal models and yeasts have demonstrated the importance of MAPs in regulating the stability and activity of microtubules, which they do by binding microtubules to each other and/or to membranes.

Gamma tubulin, originally identified in yeasts and recently in the fern, Anemia, by a group of German scientists (in Prof Schraudolf's laboratory, Ulm University), has affinity for β tubulin and is the best candidate for a plant MAP found so far.

The German group has provided a copy of the cDNA for γ tubulin to Dr Wasteneys and his colleagues Prof Gunning (RSBS) and Dr Marc (University of Sydney). They plan to make γ tubulin protein (from the cDNA) and microinject it into living cells, to try to determine its function in plant cells.

Dr Wasteneys is also screening Arabidopsis mutants to find microtubule defects (see Cellulose, the most abundant and elusive biomolecule, page 11) in collaboration with Dr Richard Williamson (RSBS). They aim to identify genes and gene products that are necessary for microtubule organisation, in order to understand what regulates the structural dynamics of microtubules and, ultimately, cell morphogenesis.

If you want to know more

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- Injury to Nitella internodal cells alters microtubule organization but microtubules are not involved in the wound response. Ilse Foissner and G.O. Wasteneys. Protoplasma (1994), **182,** 102-114.

- You are welcome to contact Dr Wasteneys via the address and phone numbers on page 2.

Dr Geoff Wasteneys.

Cellulose

the most abundant and elusive biopolymer

Every year, some 10^{11} tonnes of the polysaccharide cellulose is produced by the world's plants, making it the most abundant biomolecule on the planet. Yet, scientists are still frustrated by their own efforts to make cellulose in the test tube. Scientific literature is smattered with articles claiming to have discovered a successful method for synthesising cellulose. Unfortunately, such claims are usually followed by a barrage of reports questioning the validity of these findings. In all instances where scientists have made such claims, only a few micrograms of the polysaccharide was ever produced — an amount that is pitifully inferior to the amount of cellulose which is synthesised naturally, by even the simplest plants.

Cellulose is the major fibrous component of the plant cell wall, a vital structure which provides a 'home' for the plant cell proper. A plant cell without a wall is unable to develop an asymmetrical shape or divide properly because the cytoskeleton alone is unable to maintain these processes. Essentially, a plant's cell wall acts as the skin, skeleton and circulatory systems do for animals. All intercellular communication between plant cells must travel through the cell wall and it is, therefore, meaningless and incorrect to draw artificial boundaries around 'the cell' which do not take into account all of the biological activities of the cell wall.

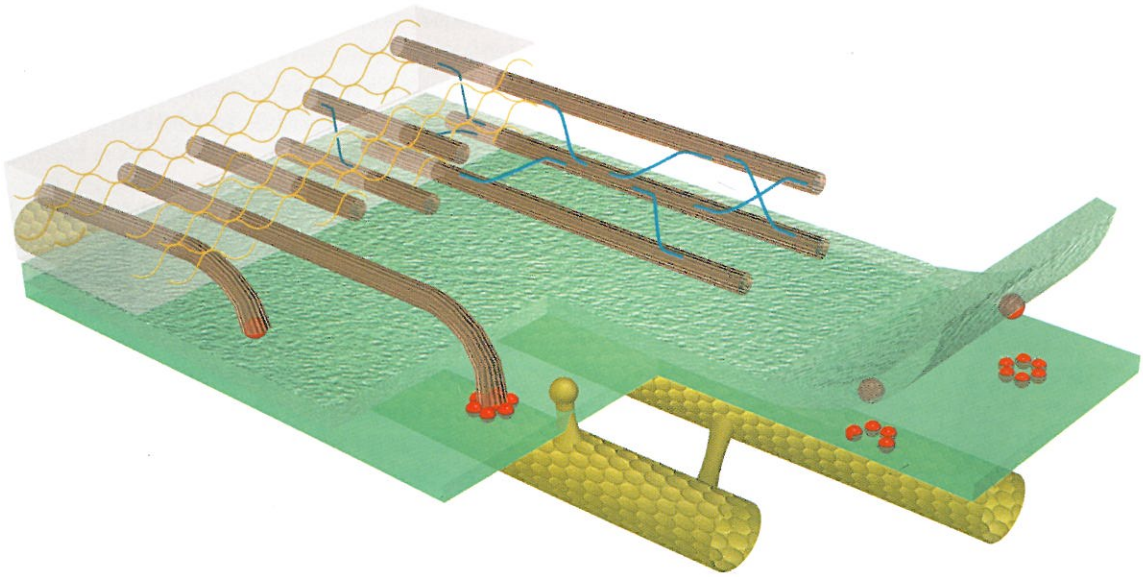

Figure 1. Schematic representation of cellulose biosynthesis occurring at terminal enzyme complexes (red), located in the plasma membrane. Newly synthesised cellulose microfibrils are extruded into the cell wall above and become extensively cross-linked with other cell wall proteins and polysaccharides. The alignment of cortical microtubules (yellow), situated below the plasma membrane (green), control the orientation of cellulose microfibrils (brown) in the cell wall (refer to The dynamic structure and structural dynamics of tubulin for more detail).

Plant cell wall research is currently in vogue with industry and cellulose synthesis on an industrial scale is a highly sought after goal. Industry clearly stands to reap considerable benefit if it becomes possible to make designer plants with increased amounts of cell wall cellulose. This might be possible if the gene or genes encoding the enzyme complex responsible for cellulose synthesis were available for manipulation. Most plant cell walls contain about 30% cellulose, an exception being cotton fibre which has a 90% cellulose content — even this amount of cellulose could be improved upon!

Dr Richard Williamson directs a research team investigating cellulose biosynthesis, based in the Research School of Biological Sciences. This research is supported in part by The Cooperative Research Centre for Plant Science, which, through two of its industry associates, Cotton Seed Distributors and North Eucalypt Technologies Pty Ltd., is providing both resources and a practical focus for the research. Rather then pursue the more traditional biochemical approach to solve this enigma, Dr Williamson has employed a classically genetic approach. He believes that, 'analysis of plants with mutations in their cellulose biosynthesis pathway could hold the key to discovering the cellulose synthase gene or genes.'

Cellulose

A molecule of cellulose is comprised of thousands of glucose (a simple sugar) monomers, covalently linked together via a characteristic β1–4 glycoside bond, giving cellulose its distinctive ribbon-like structure. Hydrogen bonds stabilise cellulose internally and link parallel ribbons of cellulose together (up to 70 cellulose chains), to form long cables of cellulose, called microfibrils. What makes cellulose so unusual is its strength.

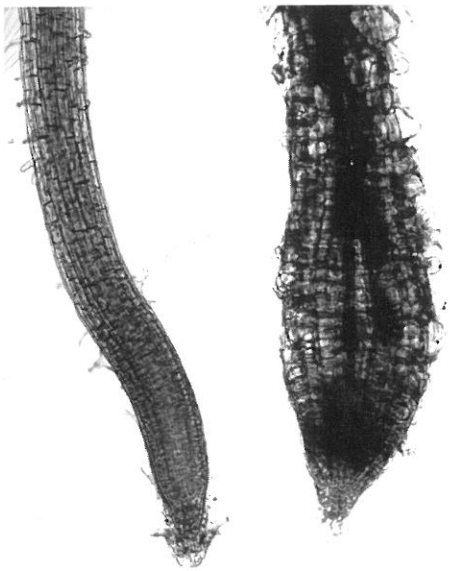

Figure 2. A comparison between a normal root tip (left) and a mutant root tip (right) which has expanded in a radial direction, due to a mutation in the cellulose biosynthesis pathway. The characteristic of root swelling was used by Dr Williamson as a marker for seedlings deficient in cellulose biosynthesis.

The structure and strength of the cell wall can be likened to reinforced concrete. To make reinforced concrete, steel rods are laid down to provide structural support and strength to the concrete poured over it. Within this structure, the weakest point is the bond between the steel and concrete. In the case of the plant cell wall's structure, parallel microfibrils are embedded in the cell wall, surrounded by a complex matrix of proteins and polysaccharides (see Figure 1). It is far more sophisticated in terms of its mechanical properties than reinforced concrete because the microfibrils are cross-linked together by smaller molecules (hemicellulose or xyloglucans). Furthermore, other cell wall polysaccharides, pectins, and glycoproteins, provide another level of structural support and complexity! The precise composition of the cell wall varies between species as well as between regions of a single plant. Due to this immense complexity, a complete description of all the molecules and their linkages does not exist.

The problem of how cellulose is produced has languished for the past 40 years. Today it is known that cellulose microfibrils are synthesised by structures called 'terminal enzyme complexes', which are localised in the plasma membrane. In higher plants, these terminal enzyme complexes are arranged as rosettes (see Figure 1). It is believed that each rosette forms a single 5 nanometre microfibril and extrudes it into the cell wall above. Multiple rosettes occur in the plasma membrane. This close proximity favours association between microfibrils to form larger fibrils in some plants.

Cellulose also plays a significant role in determining a plant cell's shape. Newly divided plant cells are generally very small and in some cases expand 100 times over their original size. The direction in which a cell may expand is governed by the orientation of the innermost cellulose fibrils. Forty years ago, the scientists Ledbetter and Porter first observed and reported that cortical microtubules exhibit a similar pattern of orientation to the innermost microfibrils. This observation has been avidly researched ever since (see article page 7). It is currently accepted that

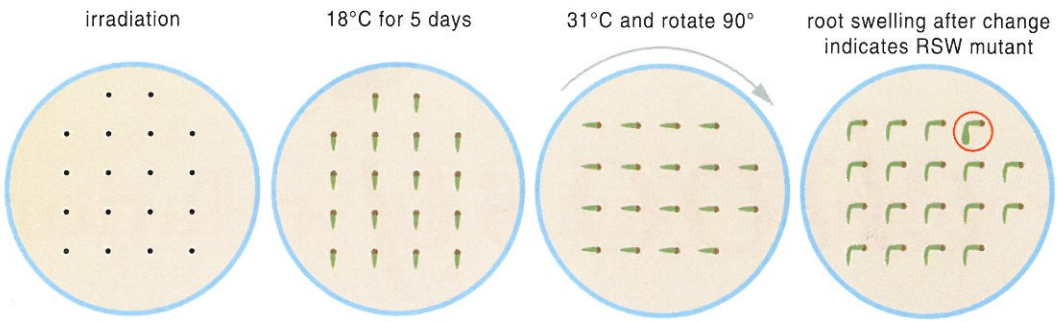

Figure 3. 1) Arabidopsis seedlings are subjected to EMS mutagenesis. 2) The mutagenised seedlings are placed on a vertical gradient and grown for 5 days at 18°C. 3) The plates are rotated through 90° and the temperature increased to 31°C. 4) The subsequent bend in root growth marks the point of temperature change. Radial swelling of root tips, beyond the bend, indicates temperature sensitive, cellulose deficient mutant seedlings or rsw (root swelling) mutants.

cortical microtubules control the orientation of micofibrils in the cell wall. Today there are many hypotheses aimed at answering what governs the orientation of the cortical microtubules!

Searching for the cellulose synthase genes

It is important to have precise and efficient tools at hand when trying to define and locate a single gene from amongst approximately 50 000 genes in the genome of *Arabidopsis* (a commonly studied plant species). This rather daunting task is being tackled by Dr Williamson and his colleagues Drs Tony Arioli and Andreas Betzner, Ms Ann Cork and Ms Rosemary Birch.

Dr Williamson's idea of generating and selecting mutant *Arabidopsis* seedlings deficient in cellulose synthesis, is certainly a novel approach. With the assistance of his team, Dr Williamson has designed some very neat experimental methods to aid his search for the cellulose synthase gene(s).

Mutant *Arabidopsis* seedlings were made by exposing seed to the common mutagen ethyl methane sulfate (EMS). EMS tends to cause more subtle changes to gene sequences (often only single base pair changes), rather than deleting chunks of the genome, as is the case with radiation. These mutagenised seeds were then spread onto agar plates and placed upright at 18°C and allowed to germinate.

As mentioned earlier, the orientation of microfibrils determines the shape of a growing plant cell. Developing root cells usually increase 10–50 times in length (because of their transversely orientated microfibrils), driving roots tips down into soil. Some herbicides work by depolarising microtubules or by stopping the synthesis of cellulose. Under these conditions, root cells, which still contain several atmospheres of pressure, expand in a radial direction rather than longitudinally. Obviously, this root swelling is deleterious to plants as it is no longer possible to maintain normal root structure. Root swelling is an ideal marker for Dr Williamson to use for rapid recognition of seedlings harbouring mutations which impair cellulose synthesis (see Figure 2).

In addition to requiring a simple marker for recognising cellulose mutants, Dr Williamson wanted to be able to control the point at which the root swelling mutation occurred. This may sound like a tall order but it is surprisingly simple, Dr Williamson selected temperature-sensitive mutants. In these mutants the defective gene only become deleterious to the plant at high temperatures. This is because the mutation destabilises the protein product, such that when temperature increases, the protein denatures. For example, temperature-sensitive root swelling mutants germinate and grow with close to normal cellulose production at 18°C and their root tips are straight. When the ambient temperature is increased to 31°C, however, the mutated protein denatures and is no longer functional, causing a deficiency in cellulose production. Under the latter conditions, developing root tips begin to swell. A simple change in temperature can, thus, control the structure of the mutated gene product, and thereby, decrease the production of cellulose.

To encapsulate this procedure; *Arabidopsis* seedlings were subjected to EMS mutagenesis, placed onto agar plates and allowed to germinate on a vertical gradient for 5 days at 18°C. The agar plates were then rotated through 90° and the temperature increased to 31°C. The subsequent bend in the growth of the root marks the time of temperature change. Seedlings which have temperature-sensitive mutations are clearly marked by radial swelling beyond the rotation-induced bend in root growth and are called root swelling (rsw) mutants (see Figure 3).

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The *choosy* relationship

Most of us are choosy when it comes to relationships. The relationship between the host flax plant (*Linum usitatissimum*) and the parasitic flax rust fungus (*Melampsora lini*) has evolved into an extremely choosy affair. It is normal for a pathogen to be host-specific, however, when it comes to flax and flax rust, the situation is excessive because a successful infection of the host will only develop when a particular strain of rust fungus interacts with a particular strain of flax plant.

This relationship is under close scrutiny by scientists at CSIRO's Division of Plant Industry and the Research School of Biological Sciences (RSBS). Their research aims to better understand both the molecular and genetic mechanisms of rust fungus infections of flax plants. The research is also linked through the Cooperative Research Centre (CRC) for Plant Science. This CRC provides a research melting-pot for scientists investigating plant-microbe interactions by uniting these otherwise separate groups and by doing so hopes to develop genetically manipulated (transgenic) rust-resistant flax plants, which have the potential to save cereal growers millions of dollars in lost yields.

Figure 1. A compatible relationship (left), between the rust fungus and flax plant, where the infection by the rust proceeds unnoticed by the plant's defence mechanisms. An uninfected flax plant (right).

Dr Adrienne Hardham heads the RSBS based team which is examining 'subcellular dynamics' during the infection of flax by flax rust and in particular, the participation of the cytoskeleton in the plant resistance response. Drs Jeff Ellis, Jean Finnegan and Greg Lawrence, based at CSIRO, have recently isolated the gene in flax that confers resistance to flax rust infection.

Flax & flax rust—an infectious old relationship

Flax is one of the oldest cultivated plants and, interestingly, it is not found growing wild. It is still being commercially produced on a global scale for fibre and oil products. Rust fungi, named after the orange-ochre colour of their spores, have existed since the cretaceous period (ca. 130-150 million years ago). It seems possible then, that rust fungi have been a major problem in agricultural productivity for centuries!

Fungi lack chlorophyll and consequently require an exogenous source of energy as well as nutrients. *M. lini* is an obligate, biotrophic parasite; that means it will only grow and reproduce on a living host. The relationship is parasitic as *M. lini* benefits at the host's expense. Because it is an obligate parasite, *M. lini* relies completely for its existence on its host.

Spores of the flax rust fungus are blown in the wind onto the surface of flax leaves. The spores germinate on the leaf and then attempt to invade and establish a close relationship with cells inside the leaf. A compatible relationship is one where the infection by the fungus proceeds apparently unnoticed by the plant's defence mechanisms and disease develops. The opposite result occurs in an incompatible relationship, where the plant's defence mechanisms recognise the invading fungus and prevent the development of disease. In a compatible interaction, the fungus is said to be virulent and the plant susceptible. In an incompatible interaction, the fungus is avirulent and the plant resistant. The development of compatible or incompatible relationships depends upon the genetic attributes of both the rust fungus and the flax plant (see Figure 1).

between flax and rust fungus

In 1956, Flor first described the evolutionary relationship between host resistance and the disease causing agent (pathogen) virulence as a dynamic equilibrium, whereby, the pathogen is neither rampant nor eliminated. Flor proposed a 'gene for gene' theory to encapsulate his finding — for every resistance gene in the host there exists a complementary avirulence gene in the pathogen. These genes generally are dominant and disease prevention (an incompatible relationship) occurs when the product of a host's resistance gene recognises and interacts with the complementary avirulence gene product of the pathogen.

This incompatible interaction between the respective host and parasite gene products initiates a cascade of events which comprise the host's defence response. The host's defence mechanisms are manifested as changes in gene expression and protein synthesis which ultimately lead to the necrosis of the host's infected cells. This plant defence mechanism, known as hypersensitive cell death, does not discriminate as to the type of invading pathogen. Pathogenic fungi or bacteria, insects or other forms of stress upon a plant may initiate the specific cascade of molecular events that culminate in hypersensitive cell death. Among other changes, alterations to the organisation of the host cell's cytoskeleton has been postulated to be an important reactive component during hypersensitive cell death.

Researching plant—microbe interactions

The cytoskeleton is a dynamic structure which responds to many different external stimuli, including gravity, wounding and stress. Dr Adrienne Hardham explains that 'rust fungi are another external factor that present a serious threat to plants.' She hypothesised that, 'if the cytoskeleton does indeed play a central role during the initiation and mobilisation of the host defence response, then changes in the arrangement of cytoskeletal elements should occur during host-pathogen interactions.'

The flax and flax rust are an ideal model for investigating plant-microbe interactions, since it is possible to innoculate a single strain of flax with either a compatible or an incompatible strain of rust fungus. Prior to Dr Hardham's research, little was known about the behaviour of the cytoskeleton during an attempted infection of flax by the fungus. To appreciate her research findings, it is necessary to be aware of the developmental stages during the establishment of a compatible and an incompatible relationship.

A compatible host-pathogen relationship

To establish a compatible relationship, the rust fungus must first penetrate the surface of the host's leaf and then invade a mesophyll cell. This occurs over a series of stages (see Figure 2a). The process begins when fungal spores land on the leaf where they germinate and a germ tube grows until it comes to a pore in the leaf called a stomate.

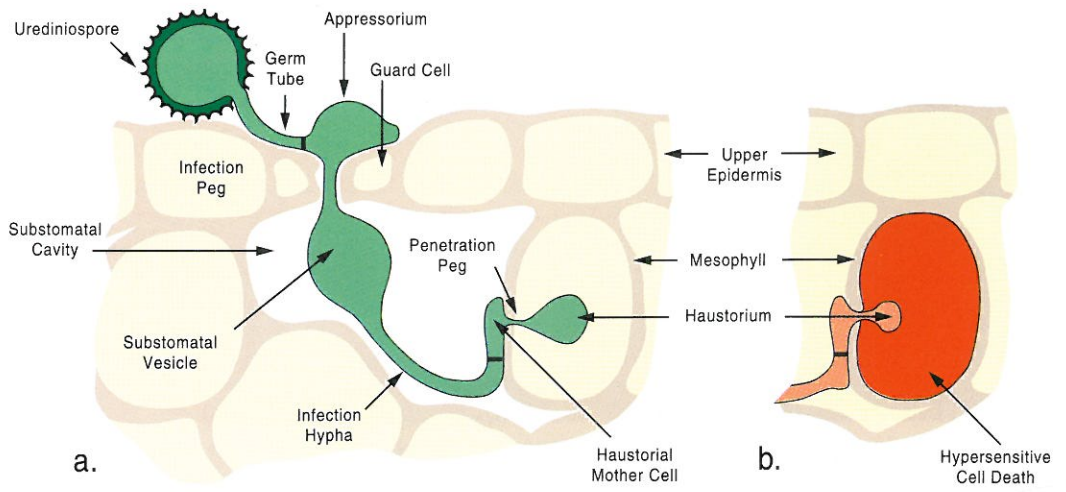


Figure 2. The developmental stages during the establishment of a compatible (a) and an incompatible (b) relationship.

The point of the germ tube then forms a cover over the stomatal pore called an appressorium. A structure called an infection peg grows from the appressorium through the stomatal pore and into the 'substomatal cavity'. Before finding a mesophyll cell, the infection peg differentiates into what's called a substomatal vesicle, which largely fills the substomatal cavity. Then hyphae, or more precisely infection hyphae, grow from the substomatal vesicle to make direct contact with a mesophyll cell. Small terminal branches, called haustorial mother cells, develop outside the mesophyll cell wall. The haustorial mother cells send out narrow penetration pegs, that are loaded with a cocktail of degradative enzymes, which, when combined with mechanical force, break down components of the cell wall. Once through the wall, the penetration pegs do not breach the plasma membrane, rather they cause the plasma membrane to invaginate as they expand to form haustoria.

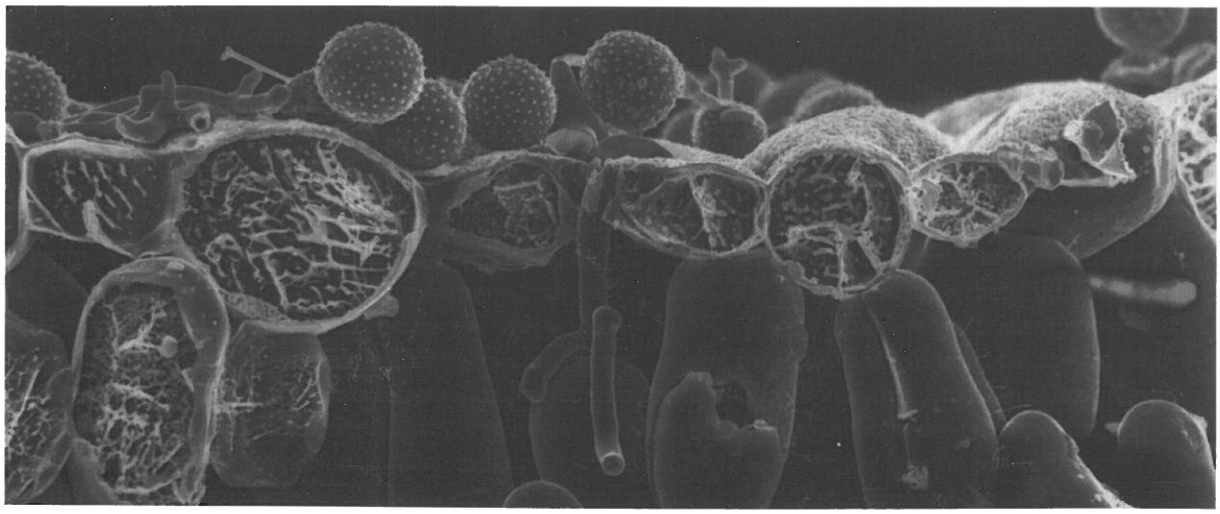
The haustorium is responsible for nutrient uptake from the host and is probably involved in signalling during a compatible flax and flax rust relationship. In highly compatible relationships, initially at least, there are no obvious detrimental effects on the host cell. However, the pathogen may be subtly directing changes in the host cell's organisation, gene expression and metabolism.

An incompatible host-pathogen relationship

The initial events that occur during the interaction of incompatible lines of flax and flax rust are the same as those observed during a compatible interaction. Spore germination, growth of the germ tube, appressorium development, entry into the host, substomatal vesicle growth and development of infection hyphae, all proceed normally and on a similar time scale in both cases (Figure 2a). Differences emerge, however, about the time that haustoria begin to form within the mesophyll cells. Fewer haustoria develop in an incompatible pairing and those that do are small and non-functional. In addition, the infected mesophyll cells undergo rapid 'hypersensitive cell death'. This necrosis of the host cell effectively kills the invading fungus (Figure 2b).

Searching for subcellular clues of early defence reactions

Dr Hardham and her colleagues are investigating how flax plants respond, at the subcellular level, to the establishment of the host-parasite interface. The experimental procedure involves attaching antibodies to actin and tubulin (immunolabelling), in flax mesophyll cells inoculated with either a compatible or an incompatible strain of flax rust. Uninfected leaves were also immunolabelled so that the normal distribution of cyto-skeletal elements in mesophyll cells could be seen. Here, microtubules, polymers of tubulin, formed transverse arrays in the cell cortex (Figure 4a). Microfil-aments, monomers of actin, were seen radiating throughout the cytoplasm from the nucleus. Mesophyll cells infected with a compatible strain of flax rust had the same cytoskeleton organisation—not surprising since the flax's defence systems doesn't mount an attack when an invading flax rust is compatible.



Dr Stuart Craig (CSIRO Plant Industry)

Figure 3. An electron micrograph of a transversely sectioned flax plant's leaf, 36 hours post infection. The invading rust fungal spores (visible on the leaf surface), have germinated and infection hyphae are present in the substomatal cavity ready to invade the surrounding mesophyll cells.

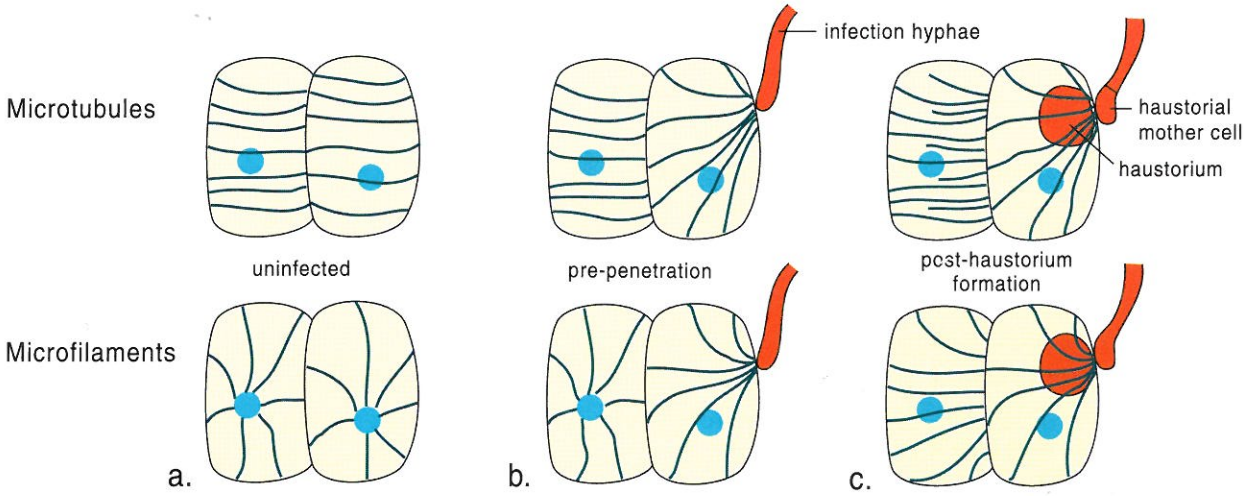


Figure 4. A schematic representation of the arrangement of microtubules (top row) and microfilaments (bottom row), during an incompatible infection. a) Normal distribution of microtubules and microfilaments. b) Both cytoskeletal elements are rearranged, becoming focused at the site of contact between the infection hyphae and the cell wall. c) An accumulation of cytoskeletal elements occurs in the walls of mesophyll cells adjoining the infected cell.

The flax's defence mechanisms, however, mount a dramatic attack upon an invading rust fungus if it is an incompatible strain, killing any mesophyll cells that are being infected. By examining the arrangement of the cytoskeleton during this event, Dr Hardham and colleague Dr Issei Kobayashi, discovered that the flax recognises and responds to the presence of an incompatible strain of rust before it breaches the cell wall. Microtubules and microfilaments become concentrated and focused at the contact site between the infection hypha or haustorial mother cell and mesophyll cell (see Figure 4b). This rearrangement of cytoskeletal elements is maintained during the attempted haustorium development, but the number of microtubules diminishes with the onset of a hypersensitive response. There were no changes to cytoskeletal elements in mesophyll cells inoculated with a compatible rust fungus; it seems that the cytoskeleton is involved in the flax plant's defence response.

In addition an accumulation of microfilaments and microtubules was observed in mesophyll cells along the walls adjoining the necrotic cell (see Figure 4c). This may be interpreted as an attempt to wall off the necrotic area and prevent the spread of infection.

Dr Hardham postulates that, 'the initial reorganisation occurring before actual penetration is likely to be induced by one or more chemical factors released by the flax rust or by the flax cells as a result of interaction with fungal factors.' She also points out that, 'the chemical signals that induce reorganisation may not be the same as those that induce hypersensitive cell death, but either way, sensing the flax rust before penetration would be advantageous for rapid activation and expression of the resistance response.'

Dr Hardham believes that the reorganisation of the cytoskeleton in infected and adjacent cells is a key factor in the flax cell's ability to resist infection. If, for example, the microtubules in the flax cells are experimentally removed, then the number of haustoria that form in the mesophyll cells in a normally incompatible interaction is greatly increased. She also believes that the cytoskeleton enables the cell to focus its defence response at the site of the invading pathogen. It is likely that the cytoskeletal elements move vesicles, and the organelles that make them, into the cytoplasm beneath the infection site. Some vesicles will contain toxins and enzymes that, when released from the cell, will inhibit fungal growth. Other vesicles will contain molecules that will greatly strengthen the flax cell wall at the point of attack, making it more difficult for the fungus to breach the wall.

While conclusive proof of the role of cytoskeletal elements in these processes remains to be uncovered, knowledge of the genetic traits which confer a compatible or an incompatible relationship will assist in elucidating the early signalling mechanisms that lead to cytoskeletal reorganisation and the defence response.

Resistance genes

It has only been in the last couple of years that scientists have managed to clone plant resistance genes—forty years after they were first postulated to exist by Flor. The proverbial search for a needle in a haystack is on a comparable scale with the scientific search for a specific gene in an entire genome, especially when the product of that gene is also unknown!

To date, five resistance genes have been cloned, including the 'rust resistance gene' or L gene. Dr Jeff Ellis explains, 'the L resistance gene has a corresponding avirulence gene in the pathogen, and it is the product of that rust fungus gene that is being recognised by the L gene product.'

Little is know about the flax rust avirulence genes or their products. Their continued presence suggests that they play a role in the biology of the fungus; they may even play a role in the infection process.

There is good reason to favour this speculation. An incompatible relationship is deleterious to the survival of the flax rust and consequently, enormous selection pressure is placed upon the fungus to lose the synthesis of that avirulence gene product, so that its spores can grow unnoticed by the host. Selection pressure is then placed upon the host flax plant to acquire a modified resistance gene. If the host successfully develops a 'counter-gene', selection pressure is returned to the pathogen. 'That's how we envisage these gene for gene relationships evolving,' says Dr Ellis.

If you want to know more

- Dynamic reorganization of microtubules and microfilaments in flax cells during the resistance response to flax rust infection. I. Kobayashi, Y. Kobayashi and A.R. Hardham. Planta (1994), **195**, 237–247.
- Molecular genetics of plant disease resiatance. B.J. Staskawicz, F.M. Ausubel, B.J. Baker, J.G. Ellis and J.D.G. Jones. Science (1995), In press.
- You are welcome to contact Dr Hardham via the address and phone numbers on page 2.

Dr Adrienne Hardham.

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'Mutations only tell you that a gene is needed, they don't tell you exactly what that gene does,' says Dr Williamson. Generating rsw mutants was only the initial stage of this research. It is now vital for Dr Williamson to be certain that the rsw mutants he has selected to analyse further are actually deficient in the machinery required for cellulose synthesis, rather than having another defect which indirectly produces the same result. In order to identify rsw seedling that are most likely to contain mutations in their cellulose synthase gene(s), he examines the ability of rsw seedlings to incorporate glucose (a precursor of cellulose) into cellulose. To do this he feeds both rsw and wild type (wt) seedlings glucose labelled with a radioisotope C^{14}. Measuring the amount of C^{14} labelled glucose incorporated into cellulose by wild type seedlings gives a standard for the amount of glucose required for normal cellulose production, making it possible, after measuring C^{14} glucose incorporated into cellulose by rsw seedlings, to express this amount as a percentage of the normal synthesis in wt seedlings. Using this method Dr Williamson and his team have found 3 rsw mutants which incorporate 40–70% less glucose into cellulose than did the wt, at 31°C.

Analysis of these three rsw mutants has, so far, shown that each mutated gene is localised to a different chromosome, providing no evidence for a cellulose synthase gene cluster. This analysis is the tip of the iceberg, as it still remains to find the precise chromosomal location of these genes so that they may be cloned and sequenced. It will then be possible to discover what part each individual gene product plays in cellulose production.

'We really hope that one of these mutations encodes a subunit of the cellulose synthase gene! They could be only one part of the cellulose synthetic enzymatic machinery, or they could be genes whose product regulates the activity of the cellulose synthase enzyme, or a cellulose synthase transcription factor,' says Dr Williamson. Furthermore, he points out, 'five years or more is a long time to wait for such an answer but if it finally unlocks the mystery of how plants make cellulose, the RSBS group will feel their efforts have been well rewarded. Besides, such long time frames are the essence of basic research, especially when dealing with major unsolved questions.'

If you want to know more

- Root Morphology Mutants in Arabidopsis thalaiana. T. Baskin, A. Betzner, R. Hoggart, A. Cork and R. Williamson. Australian Journal of Plant Physiology (1992), **19**, 427-37.
- The biochemistry of cellulose synthesis. D. Delmer. The Cytoskeletal Basis of Plant Growth and Form. (C. Lloyd ed.) Academic Press. (1991) pp 101.
- You are welcome to contact Dr Williamson via the address and phone numbers on page 2.

Dr Richard Williamson

Rhizobium

the refined parasite of legumes or cunning burglar

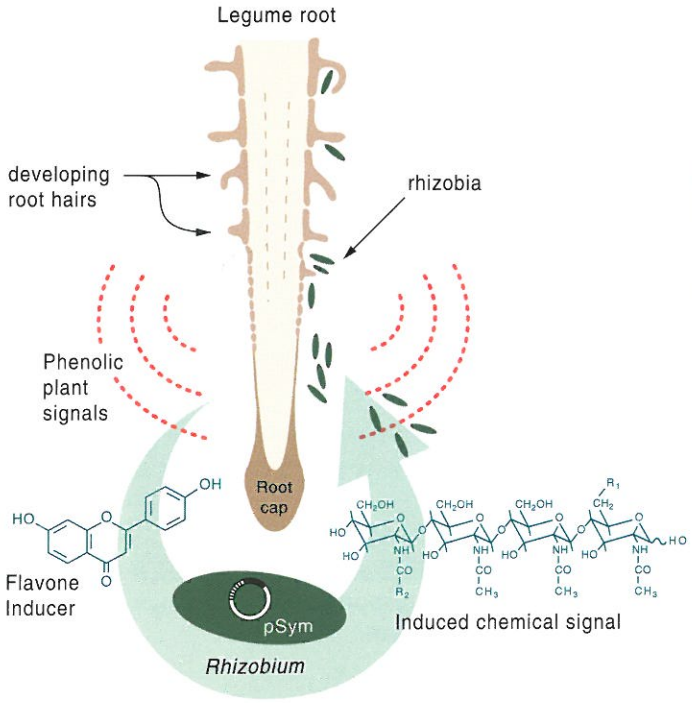
A wide range of relationships exist between plants and bacteria. Extremes of this range include 'pathogenic' relationships (where the bacteria benefit at the plant's expense) and 'symbiotic' relationships where both the bacteria and the plant benefit (see *The Choosy relationship between flax and rust fungus*). Ultimately the most advanced plant-interacting microbes, called symbionts, not only exploit their host without damage but they actually provide them with some sort of advantage.

Prof Barry Rolfe, of the Research School of Biological Science, has been investigating how the cell surface mediates the crucial interactions between the bacteria, rhizobia, and their legume hosts. The answers to questions such as how do rhizobia control, mask and/or manipulate the host's normal defensive reactions to an invading microbe, are intimately associated with cell surface signalling. Understanding such processes becomes even more intriguing when it is considered that preknowledge of a potentially beneficial relationship, on behalf of the host, does not exist.

Rhizobia

All nutrients required by plants, apart from nitrogen, are absorbed directly from the soil. Nitrogen that has been incorporated into living systems has ultimately been derived from the atmosphere. The only organisms capable of 'fixing' nitrogen ie. converting it into a form useful to plants are certain cynobacteria and eubacteria. Rhizobia, a species of cynobacteria, can induce 'nitrogen-fixing nodules' on particular legumes. Nitrogen fixation, usually involves converting atmospheric nitrogen into ammonia and is an energetically expensive process. Hence, the nitrogen-fixing *Rhizobium*-legume symbiosis is one of the most important relationships in agriculture globally.

Each year, the global fixation of nitrogen into plant-useable ammonia by rhizobia, amounts to approximately 75 million tonnes. This is equivalent to 160 million tonnes of urea (a commonly used fertiliser), or to roughly $65 billion US dollars, delivered *gratis* into the global economy.



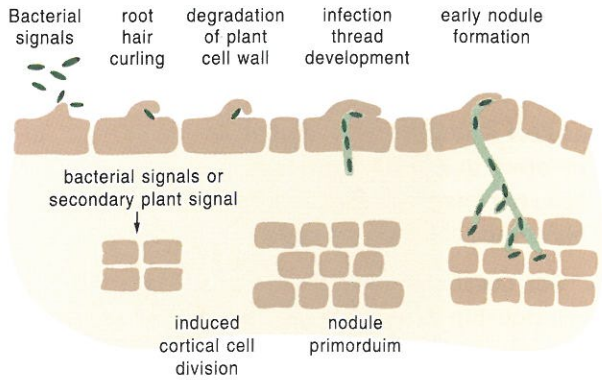


Figure 1a. A schematic representation of molecular signalling between a legume host and rhizobia, during the development of an infection and symbiosis.

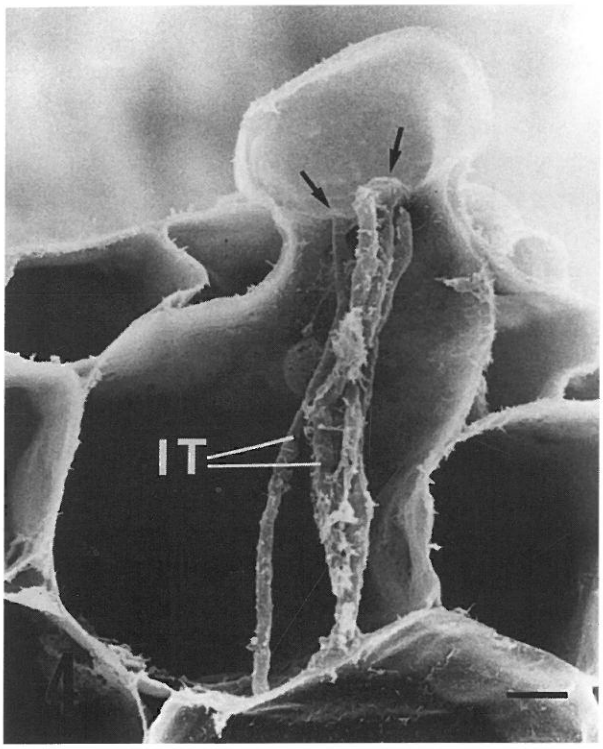


Figure 1b. An electron micrograph showing the infection thread (IT).

There is great commercial interest in developing more competitive strains of Rhizobium that have an enhanced ability for nitrogen fixation. It is not surprising, therefore, that scientists from CSIRO's division of Plant Industry, the Department of Biochemistry and Molecular Biology (Australian National University) as well as Prof Rolfe and his colleagues at RSBS, are involved in Rhizobium research and that this research has unravelled many of the complex plant-microbe interactions.

The development of a successful symbiotic relationship involves precise and highly regulated interplays of signalling and gene expression between the potential partners. Rhizobia cells contain only minimal genetic material, one molecule of DNA and some strains have up to 5 plasmids (small fragments of DNA). Despite this, Prof Rolfe says that, 'a couple of hundred genes may be being manipulated by the invading Rhizobium,' during the infection process.

Plasmids lend themselves to genetic manipulations and by a stepwise process of eliminating plasmids from Rhizobium, scientists found that a particular plasmid, the sym (for SYMbiosis) plasmid carried the genes which direct nodulation. Three groups of genes from the sym plasmid, are intimately involved in the nodulation process. They are the nod genes (encoding nodulation gene products), the nif genes (encoding the nitrogenase enzyme) and fix genes (encoding regulatory factors of nitrogen fixation).

How the partnership evolves

In most legumes, the site of infection is restricted to the root-hairs. Flavonoid compounds are small phenolic molecules produced by plants which are secreted in high concentrations from the emerging root hairs. Flavonoids stimulate the expression of Rhizobium nodulation genes. Essentially, the flavonoids attract the bacteria to the emerging root hairs, whereupon the nodulation gene products make chemical signals that begin to modify the normal development and metabolic state of the root hair. The root hair distorts and begins to curl over, enveloping the Rhizobium (see Figure 1a).

A structure called an infection thread facilitates entry by the bacterium into the root. The infection thread is composed of newly synthesised plant cell wall and membrane material, inside which the rhizobia divide and proliferate (see Figure 1b). In some respects, the formation of the infection thread is similar to a standard defence response that a plant has to an invading pathogen — ie. it 'walls off' the microbe to control the spread of infection. In the case of Rhizobium, however, the bacterium has control of, and mutes the plant's defence response. Chemical signals emitted from Rhizobium can redirect the metabolism of cells that are three or more cell layers away! Cells of the root cortex begin dividing in anticipation of the Rhizobium invasion and the infection thread is laid down between and through cells, without triggering an adverse plant defence response, until a target cell is reached and here the bacteria are released inside. The invaded cells increase in size and the final root outgrowth is called a nodule (see Figure 2). When the symbiosis is complete the bacteria provide the plant with useable nitrogen and the plant provides the bacteria with shelter, amino acid precursors, energy and other nutrients. If the bacteria fail to fix nitrogen, the plant can mount a pathogenic response against them, thereby eliminating them.

Recognition and the cell surface

Carbohydrates (CHO) are primary markers for cell recognition (see attachment *Molecules of the Cell Surface*, page 22). In the late 1960's CHOs were recognised only as an energy source and as structural material (eg. cellulose and chitin). It soon became evident, however, that the structural diversity of CHOs provided an enormous advantage for information storage, especially when compared to proteins. It has been suggested that proteins are simply a platform for presenting different sugars. Most proteins and peptides exist is association with CHO-side chains, and are called glycoproteins. The presence of CHOs on proteins profoundly influences the way in which they interact with other molecules.

The extracellular matrix is a dynamic assemblage of proteins, glycans and glycoconjugates that are mediated by CHO-CHO and protein-CHO interactions (see Figure 3). This assemblage is so complex that it is sometimes difficult to say where the cell ends and the surrounding environment begins. It is known that molecular interactions in the extracellular matrix have a profound effect on the underlying cytoskeleton and cell biochemistry.

Prof Rolfe and his colleagues began investigating the role of cell surface molecules in the initial interaction between rhizobia and its host, seven years ago. He hypothesised that since the initial interaction between the two organisms occurred at the cell surface, molecules there may be involved in determining whether or not an attempted infection was successful.

Prof Rolfe wanted to identify the role of exopolysaccharides (EPS), one of the many cell surface CHO, to see whether they were involved in specific adhesion of rhizobia to the root hair surface or if they had some other, active role in root infection.

Most species of rhizobia are typified by their strict host specificity, exceptionally, the *Rhizobium* sp. strain NGR234 possess a broad host range. Prof Rolfe and his colleagues used a mutant NGR234 strain, which produces little or no EPS (an Exo^- mutant) to investigate the response of different hosts to this defect.

Analysis of a number of NGR234 derived Exo^- mutants showed that they were unable to induce nitrogen-fixation nodules, on all of their hosts. Examination of nodules induced by Exo^- mutants showed that on some plants, the nodules were poorly developed. Prof Rolfe explains that, 'these mutants cannot induce the correct CHO-mediated signals for induction of successful nodulation.'

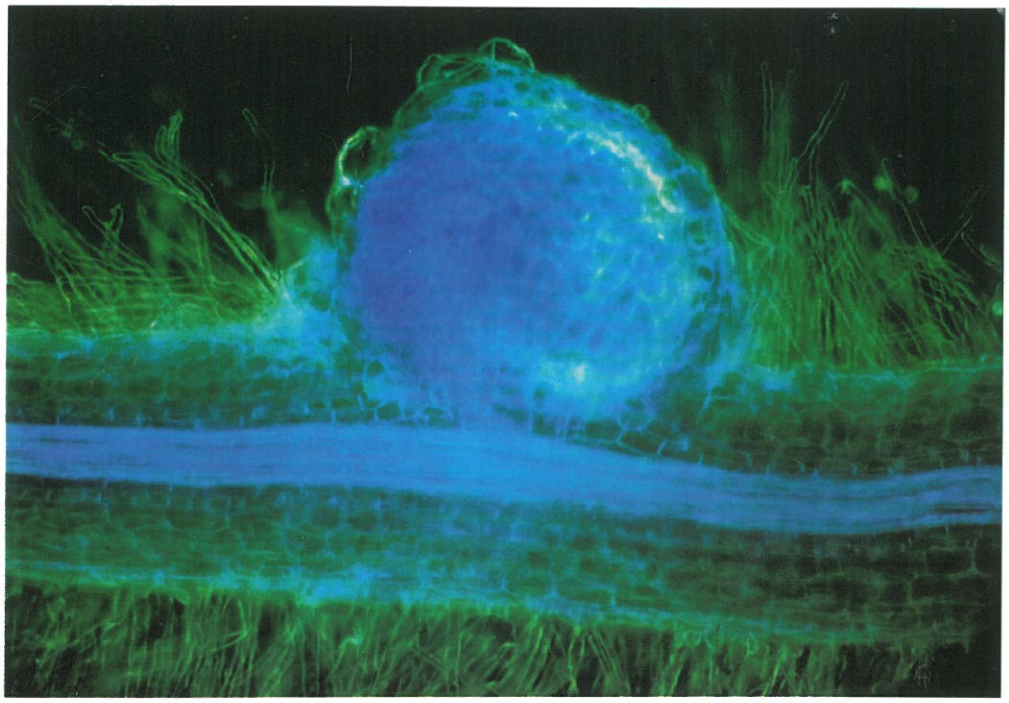
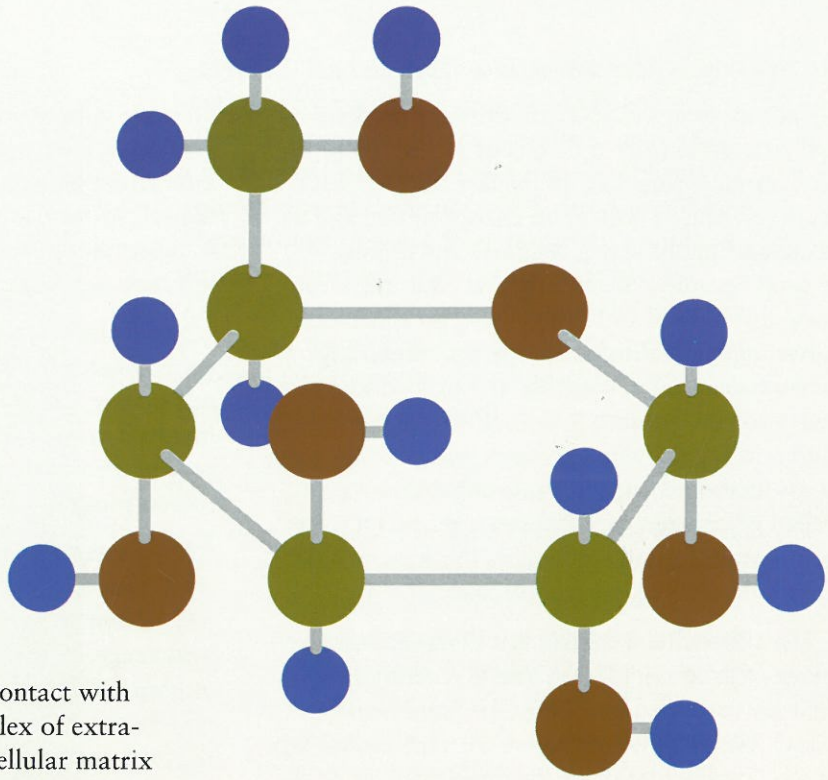


Figure 2. The final root outgrowth, seen here, is called a nodule.

Finding out: Molecules of the Cell Surface



A cell, whether isolated or in contact with others, is surrounded by a complex of extracellular components, the extra cellular matrix (ECM). The ECM and cell surface of both plant and animal cells have developed into highly specialised and dynamic assemblages of proteins, glycans (sugar molecules) and glycoconjugates (sugar containing molecules), which mediate numerous vital functions of the cell. Many of these functions, ranging from mechanical integrity to defence, are mediated through highly specific carbohydrate-carbohydrate, protein-carbohydrate and membrane protein interactions. These interactions have the most profound effects on the underlying cytoskeleton and biochemistry of a cell, both through structural interaction and through transduction, across the plasma membrane, of signals initiated by the protein-glycan interactions in the outer ECM.

What are some of these molecular components of the ECM and how are they organised? Most of the things that cells can do are done by proteins, long chains of amino acids, which are ordered according to a gene. The chain of amino acids can fold into a number of different shapes and it can have additional small components attached to it such as sugar molecules. Sugars are small molecules comprised of carbon, hydrogen and oxygen in a specific ratio $C_nH_{2n}O_n$. They can be attached to one another to make larger molecules. For example, sucrose is a disaccharide (two sugar chain), longer chains (3—12 sugars) are called oligosaccharides, while larger arrangements are referred to as polysaccharides.

Sugars attached to proteins (glycoproteins) are found throughout biology and have profound effects on the final shape, structure, specificity and function of the protein involved. The attached sugars are not necessarily linearly arranged; they can be linked to each other in all sorts of ways. This is what makes them very complicated to study. For example, while 4 different amino acids can combine to form 24 different protein fragments, 4 different monosaccharides can combine to form 35,560 tetrasacchides! The extent that cells use this near-infinite complexity of sugar chemistry in their biological functions is only now becoming apparent, thus fuelling the rapid growth of the new field of glycobiology.

Another feature which makes the cell surface so interesting and difficult to study is the dynamic nature of this structure. Perhaps it might best be thought of as an ocean, with many floating rafts on it, which can continually assemble and disassemble into millions of unique structures. It is this dynamic organisation that can provide specific spatial and temporal functions for single cells or groups of cells in a tissue. The complexity of cell surface components and sugar chemistry provides a huge challenge to glycobiology, but the basic knowledge gained will have immense applications to all agricultural, veterinary and medical sciences.

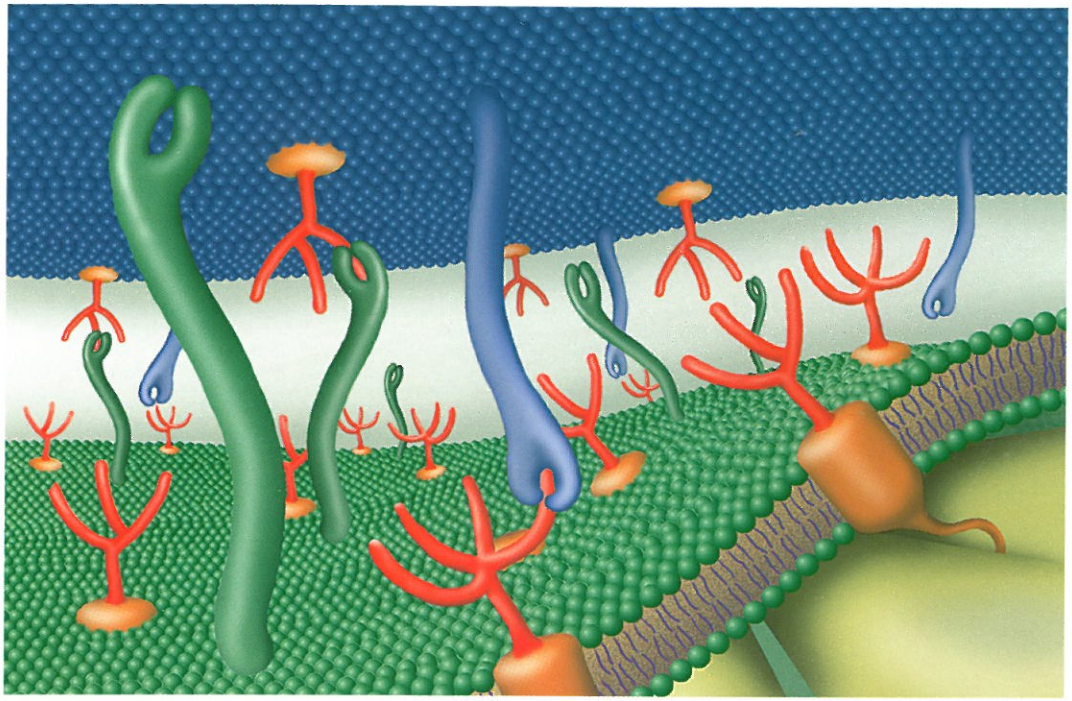


Figure 3. An interpretation of the complex interactions occuring at the cell surface.

The functional role of EPS was demonstrated by another series of experiments. The NGR234 derived Exo^- mutants were coincubated with either; 1) an Exo^+ strain unable to nodulate, 2) purified EPS from the parent strain and 3) the oligosaccharide repeat units required to synthesise EPS. The addition of any of these treatments to the Exo^- mutants restored their ability to induce nitrogen fixing nodules, on all hosts.

Prof Rolfe says that, 'this specificity of EPS action indicates that they [EPS] have more than a simple passive role of masking determinants on the *Rhizobium* surface. It is possible that EPS molecules may be regulators of specific plant functions, such as growth, differentiation and disease resistance. It is clear, however, that EPS have a specific role in effective nodule formation, they must be part of the molecular signalling between the bacterium and the host plant.'

It is known that once a host becomes infected with rhizobia, it reacts to avoid over nodulation by a type of auto regulation. It appears that molecules of the class of cell surface CHO called cyclic ß-glucans may be involved in this phenomenon. Evidence for this hypothesis came from studies where the addition of exogenous cyclic ß-glucans to *Rhizobium* inoculated legumes, enhanced both the number of nodules and the kinetics of nodule formation. More research is required to clarify whether the cyclic ß-glucans and other cell surface CHO, act directly or indirectly during the process of nodulation.

Work by Prof Rolfe and colleges has clearly demonstrated the importance of CHOs in cell signalling and identified specific roles for cell surface CHOs in the symbiotic relationship between rhizobia and its host legume. This area of research is still in its infancy and holds great promise for the future.

If you want to know more

- Genetic analysis of legume nodule initiation. B.G. Rolfe and P.M. Gresshoff. *Annual Review of Plant Physiology. Plant Molecular Biology* (1988), **39**, 297–319.
- Exopolysaccharide production in *Rhizobium* and its role in invasion. J.X. Gray and B.G. Rolfe. *Molecular Microbiology (1990)*, **4**, 1425–1431.

- You are welcome to contact Professor Rolfe via the address and phone numbers on page 2.

Professor Barry Rolfe.

FIRST... TO LEARN THE NATURE OF THINGS

This headline, a widely accepted translation of the Latin motto from the 'arms' of the Australian National University, sums up all that drives basic research and the advance of knowledge. Our universities have emerged as major instruments to hone the skills of enquiry and thus maintain the cutting edge of new knowledge. However, they can only do so in the long-term if a culture of enquiry is firmly embedded in society as a whole. This requires a better public understanding of the processes of scientific enquiry, a point that I made on the back page of *Biologic* in July 1993.

Decades ago, the need to promote greater public understanding of science was foreseen by research leaders in learned academies, such as The Royal Society and the Australian Academy of Science. Now, even governments see the need. In 1995, in Britain alone, $25 million will be spent on this quest. Improved public awareness of the importance of science in every aspect of life in Australia seems likely to be high on the agenda for Senator Cook's Innovations Statement next August.

According to a recent editorial in *Nature*, the preeminent journal of frontline research in the natural sciences, improved public understanding begins by implanting enthusiasm for science in young people. Indeed, the bottom line is seen to be *'root and branch reform of the interface between schools and universities'*.

Biologic works at this interface to increase awareness of some of the latest Australian research in biology among science teachers, and already we have had most encouraging feedback. Soon its text and images will be available in an interactive format so that teachers may devise question-answer pathways, at the keyboard, through each article. I hope we can strengthen these partnerships and help implant a culture of enquiry among students, and recognition of the Nation's achievements in science.

There are other ways RSBS hopes to improve understanding of science. For example, last January staff were engaged in some experiments with teachers at the summer science school held in ANU, using themes in the last issue of *Biologic*. This will become a regular summer activity, and John Dash in the ANU Centre for Continuing Education (Phone (06) 249 0778) can provide information on future summer schools.

The Cooperative Research Centre in Plant Sciences, in which this Research School is a major partner, also maintains a hands-on research experience with plants - the 'Green Machine'-for school parties visiting the Federal Capital. A science teacher, Graham Smith (Phone (06) 249 5879), has been seconded from the ACT Department of Education to assist.

The CSIRO Student Research Scheme, which began in Canberra in 1982, is now a national program that allows senior secondary students to participate in appropriate research projects under the supervision of practising scientists in laboratories of CSIRO, this Research School and other institutions. In the ACT and region, Dr. Mary Webb is the scheme coordinator (Phone (06) 276 6589, Mon-Wed) and information is available from CSIRO Education Programs in other cities.

While working with teachers to improve the public understanding of science, we also have to work to ensure our research stays at the cutting edge. This is done by peer review, the mechanism that has evolved to maintain standards of excellence in research. It seeks expert opinion about the quantity and quality of research from one's competitors. Like all assessment processes peer review is continually subject to scrutiny. Last year it was even endorsed by a Congressional Committee in the United States. The third review of RSBS last February was headed by the Chair of the Swedish National Research Council.

The impact of our research, some of it described in the papers listed in the boxes at the end of articles in *Biologic*, was also compared with the top five other institutions for biological research in Australia. The review panel found that *'RSBS is the premier source of fundamental biological research in Australia'*, and commented on it as *'an exemplary mechanism for the transfer of these ideas to the larger Australian scientific community.'* Obviously, *Biologic* was a persuasive part of the evidence that led to this latter statement by our peers.

Since then, the panel's conclusions have been confirmed by the highest peer review of all, the election of two RSBS Professors, Graham Farquhar and Mandyam Srinivasan, to The Royal Society of London, and The Australian Academy of Science, respectively. Recognised as being among the first to learn the nature of things, both researchers have recently contributed to *Biologic*, and to public understanding of science, with articles on global change and bee vision. These articles may help you to gauge what it takes to make the top 40 in the English-speaking world (outside the US), or the top 12 in Australia, in all fields of research in natural science. Public understanding of the strength of Australian science will have improved when these accolades are recognised as no less an achievement than the closing partnership of the Waugh twins on the 1995 Windies tour!

Barry Osmond