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Towards the Development of Heartwood-free Eucalypts

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Prepared for the

Forest & Wood Products
Research & Development Corporation

by

L. Wilson and G Bossinger

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Introduction

Significance of heartwood

Heartwood exists as a central column within the stem of many woody tree species (Figure 1). It is a developmental adaptation providing two layers of static defense against invading micro-organisms comprising dispersed toxic polyphenolic extractives, which act as chemical barriers, and in many angiosperms, including all commercially important *Eucalyptus* species tyloses (Chattaway, 1949), which act as physical barriers limiting access to the transpiration system. Tyloses also provide a dynamic defense against invading micro-organisms as part of wound response in sapwood (Beckman, 2000). While tyloses are important for the long-term survival of trees they represent a mixed blessing for the forest industry, where on the one hand they contribute to wood durability but on the other limit permeability and therefore increase the cost of wood processing. As stems of commercial size may contain in excess of 50% heartwood whose permeability may be less than 1% that of sapwood even modest gains in permeability will significantly reduce processing costs (for drying, pulping and preservative treatment) through reduced chemical usage and decreased intractable waste. Furthermore, increasing permeability of stems of less durable/less valuable species will facilitate preservative treatment allowing their use in applications presently requiring naturally durable species. Our project was therefore directed at exploring opportunities for utilising molecular tools to enhance permeability of heartwood in eucalypt stems.

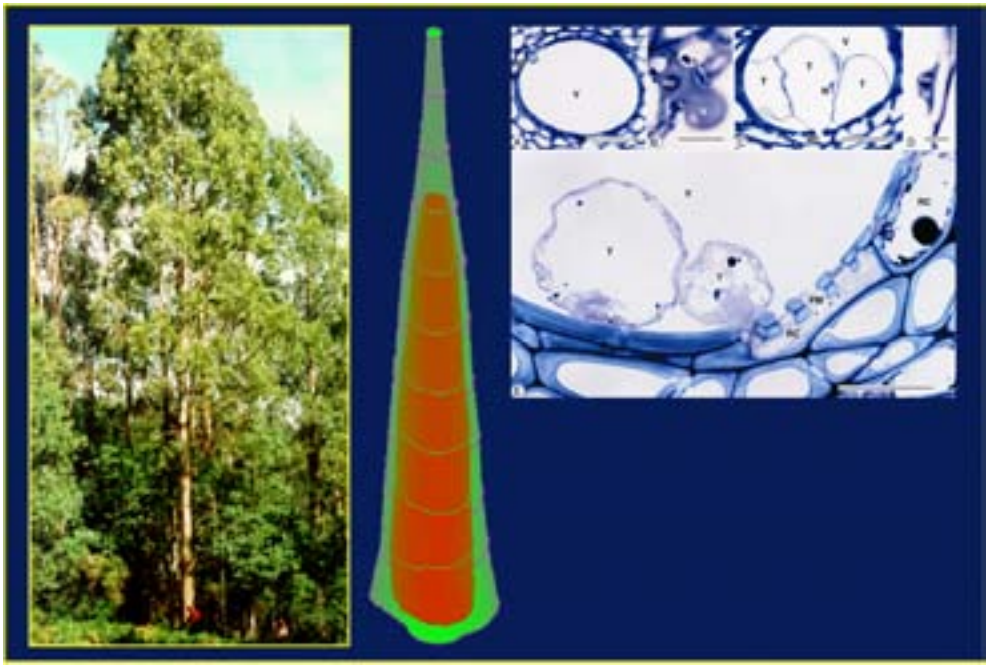

Figure 1 A 20-year-old *Eucalyptus nitens* tree (Strezlecki Ranges, Victoria) and a 3-dimensional reconstruction of the stem, including heartwood, from 0-90% height. Heartwood was 50% of volume of the stem.

Figure 2 Transverse sections. A-Tylosis emerging from vessel, B-High power of A, C-3 tyloses in a vessel, D-High power of nucleus, E-Developing tylosis emerging from ray cell. The nucleus is visible at the base of the tylosis

The nature of tyloses

Formation of tyloses from vessel-associated ray cells, which is considered the final differentiation stage of ray cell development, begins in the sapwood/heartwood transition zone (transition zone from here on) just after release of polyphenolic extractives from non-vessel-associated ray cells into the surrounding xylem. Figure 2 presents some histological images of tylosis development. Figure 2A shows the earliest stage of tylosis development with the nucleus of the ray cell emerging into the vessel lumen. This is shown at higher magnification in Figure 2B. Figure 2C shows three well-developed tyloses in a vessel lumen. The middle tylosis, which is still alive, contains a nucleus which can be seen at higher magnification in Figure 2D. Figure 2E shows a high magnification image of a tylosis emerging from a ray cell with the nucleus visible at the base of the tylosis. Figure 3 shows many synchronously

developing tyloses and associated ray cells in an induced tylosis stem section (discussed later). This image clearly illustrates the capacity of tyloses to limit permeability by blocking vessels.

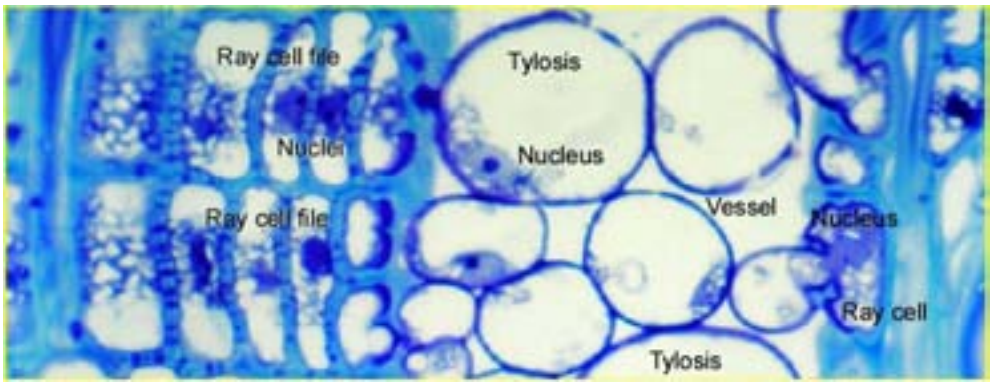

Figure 3 Radial longitudinal section from induced tyloses stem sample. Synchronously developing tyloses in vessel. Well preserved nuclei and cytoplasm can be seen in tyloses and ray cells. Sample fixed using continuous flow system.

Results

The project focussed on the molecular basis of tylosis formation and how an understanding of this might enable tylosis formation to be regulated. Crucial steps in this process were:

- Development of cDNA libraries for the study of gene expression during tylosis formation
- Development of *in vitro* systems to study gene expression during tylosis initiation
- Identification of molecular pathways involved in tylosis formation.

cDNA libraries

Construction of cDNA libraries proved far more challenging than anticipated, for two reasons. Firstly, because transition zone ray cell differentiation activity is asynchronous and the presence of differentiation mRNA is only transitory, insufficient mRNA was available for cDNA library construction; and secondly, because a large mass of xylem, containing only a very small proportion of differentiating ray cells needed to be pulverised to a fine powder under liquid nitrogen to preserve RNA for extraction.

Synchronising ray cell activity

The problem of asynchronous ray cell activity was solved by using wound reaction to stimulate a rapid heartwood-like response, including extractive release and tylosis formation, in outer sapwood This is a well documented response following physical injury (Schmitt & Liese 1994) and in stems of trees after felling (Murmanis, 1975; Brennan *et al.,* 1995). Our trials confirmed that extensive synchronised tylosis formation could be induced in outer sapwood of discs cut from stumps of 12-year-old *Eucalyptus nitens* trees whose crowns were removed one week previously (Figures 4 & 5). Stereomicroscope observation of tylosis structure and distribution showed a gradient from well developed in outer sapwood, just under the cambium, to earliest development in the third and fourth annual rings from the cambium. There was also a gradient of decreasing development down the stem, with tylosis development ceasing about 400 mm below the initial cut in the stem. The tree described here was sampled during July when maximum temperatures did not exceed 10^{O}C. Trees sampled in December when maximum temperatures exceeded 20^{O}C showed the same development in only 2 days.

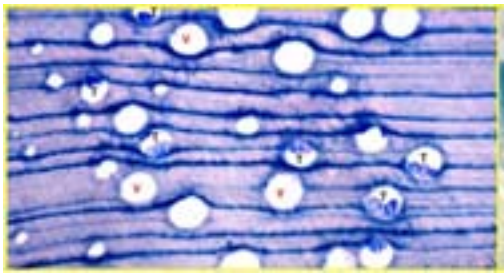

Figure 4 Tyloses in vessels. T–Tylosis, V–Vessel

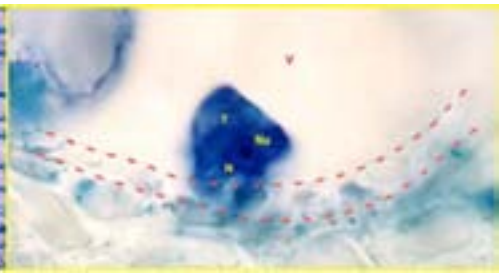

Figure 5 Tylosis entering vessel. N–Nucleus Nu–Nucleolus. T–Tylosis, V–Vessel

Pulverising xylem samples

Pulverising animal tissues and soft plant tissues is performed relatively easily using a mortar and pestle. This was not possible using xylem tissue that assumed the appearance and hardness of stone when frozen in liquid nitrogen. Small scale pulverising of hard tissue can be done with coffee grinders but these are too fragile for continuous use. The problem was solved by the development of the "Xylem Xapper" (Wilson *et al*., 2004). The device was based on the design of a kitchen blender and comprised an Hitachi M 12V router, a stainless steel pulverising tool, a stainless steel vacuum flask, an MDF stand and an MDF safety shield (Figures 6 & 7).

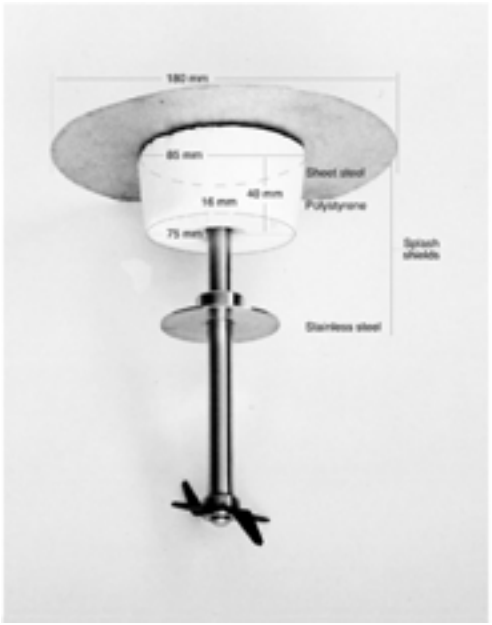

Figure 6 Pulverising tool

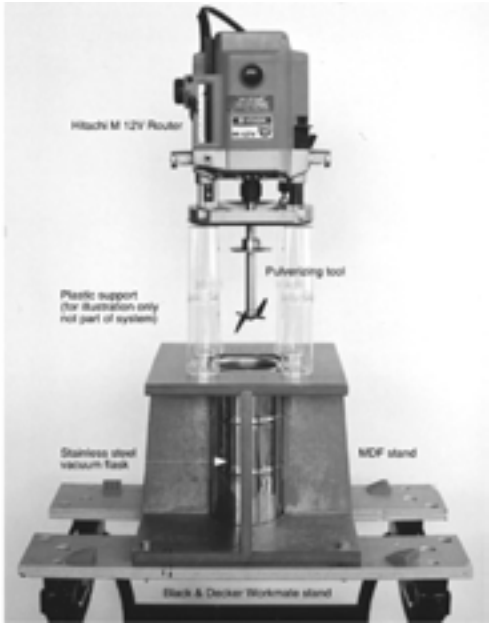

Figure 7 Components of the "Xylem Xapper"

For sampling and pulverising, segments 500 mm long, from 1.5 to 2 m above ground, were cut from normal and experimental stems of 5 m high, 12-year-old *E. nitens* trees growing at the University of Melbourne, School of Forest and Ecosystem Science site at Creswick, 120 km west of Melbourne. Discs 10 mm thick were cut from the segments, the bark was removed and the discs were immediately frozen in liquid nitrogen. All cambial tissue and developing xylem was removed (to avoid contamination with non-tylosis RNA) then the outer two annual rings of each disc were chipped off with a chisel under liquid nitrogen into pieces measuring about 10 x 10 x 5 mm. Chips were either pulverised immediately or stored in lots of about 100 g in freezer bags at -70^{o}C. For pulverising, 100 g of chips was added to liquid nitrogen in the vacuum flask and the router was run at maximum speed for 2 minutes. The powder was either used immediately or stored at -70^{o}C. RNA extraction and cDNA library construction were performed according to standard procedures with some modifications (Wilson *et al*. 2004).

In vitro *systems*

Transition zone ray cell development has been little studied *in vivo* simply because the living cells are inaccessible to investigation. Some progress has been made with *in vitro* systems (Leitch *et al*., 1999) but

these are still limited by the woody nature of the samples. We devised two *in vitro* systems: ray cell stacks and a continuous flow system. Potentially, the ray cell stack system provides the basis of a wood-free pure culture system in which communication between cells can be manipulated, cells can be transformed and cellular processes can be monitored microscopically. Both systems are, as yet, at an early stage of development. Characterisation of media suitable for initiation, maintenance and inhibition of heartwood like changes is likely to prove challenging as the *in vivo* activity of these cells is not only influenced by a variety of environmental, seasonal and hormonal changes but also by large changes in osmolality. Work on the ray cell stack system was discontinued because of the expected long development time and the focus on investigating tylosis expansin activity.

Continuous flow in vitro system

The continuous flow system is based on a four channel peristaltic pump which is able to circulate experimental reagents through the vascular system of isolated stem segments at rates between 3 and 30 ml per hour (Figure 8). The system has many potential applications for the study of ray cell development including: initiation of tyloses, study of processes leading to extractive synthesis and release, transformation of cells and morphological studies. So far, the continuous flow system has been used for fixation of both normal outer sapwood and sapwood in which tylosis formation was induced *in vivo* and provided standards of preservation for light and electron microscopy that could not be achieved by conventional fixation. An example of this is shown in Figure 3 where numerous tyloses can be seen in a vessels, some of them just emerging from ray cells. Fixation quality is excellent, with nuclei visible both in tyloses and ray cells. This image also provides confirmation of the synchronous nature of tylosis formation that can be induced by wounding.

Figure 8 Components of the continuous flow *in vitro* system

Ray cell stacks

For this project, it was thought that ray cell protoplasts extracted from developing xylem might provide a useful system for investigating ray cell development. Developing xylem is the wood just underneath the bark and contains active cells with recently formed secondary walls. These can be scraped off and collected in spring when the tree is growing rapidly. Using developing xylem from 12-year-old *Eucalyptus camaldulensis* trees (School of Forest and Ecosystem Science site at Creswick) we found that ray cell protoplasts could be readily extracted using the method of Leinhos & Savidge (1993) but as in experiments of these authors their long term viability was found to be minimal. Consideration of the literature (Raff *et al*., 1993; Ameisen *et al*., 1995) suggested that, in animal systems at least, isolation of cells from each other could induce (programmed) cell death by preventing exchange of growth factors. In plant cells *in vivo*, a wide range of substances including photoassimilates, ions, growth regulators, mRNA and proteins (Heinlein, 2002) are transported between cells through an extensive network of

plasmodesmata (fine cytoplasmic channels connecting cytoplasm of adjacent cells). During protoplast preparation, hyperosmotic solutions are used to shrink protoplasts away from secondary walls. This procedure unavoidably disrupts plasmodesmal links between cells resulting in cell isolation. Although other plant protoplast systems are sustainable when these links are disrupted, for example those produced from leaf mesophyll cells, we suspect that in ray cells isolation leads to cell death. Another related and more intractable limitation to ray cell viability is wound reaction which, in addition to activation of an array of defensive phytotoxins, involves massive cell death. By chance, a solution was found to this problem. It was observed during protoplast preparation that incomplete digestion of developing xylem released large numbers of ray cell stacks (Figures 9 & 10) consisting of intact clumps of two to more than thirty ray cells broken from the ray parenchyma system. Viability of cells in these stacks was tested using a differential scanning calorimeter (Calorimeter Sciences Corporation, Spanish Fork, Utah, USA). This device detects cell respiration as heat output from samples within sealed ampoules and showed that cells in ray stacks were viable for up to 3 days.

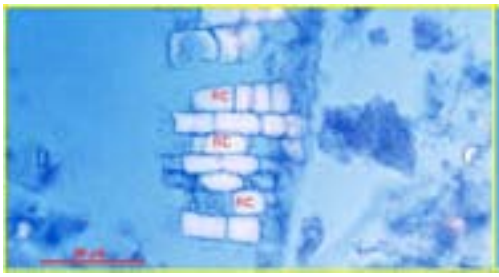

Figure 9 Ray cells in stacks, RC–ray cell

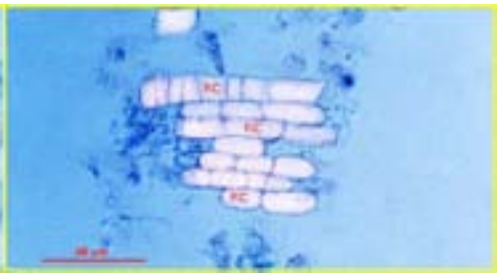

Figure 10 Ray cells in stacks, RC–ray cell

Protoplast extraction device

Enzymatic extraction of free cells from plant tissues is an unavoidably traumatic procedure in which large numbers of cells die from mechanical injury and toxic effects of enzymes. A protoplast extraction device (PED) was constructed in an earlier study (Figure 11 - Wilson 2002) to minimise these effects and was used to extract ray cell stacks. The central element of the device is the plastic PED shaft which is fitted with an array of chrome spikes to support the developing xylem sample. Stacks freed from the developing xylem by enzyme activity drop to the bottom of the cylinder without further mechanical damage. The power supply for the motor drive is fitted with a tachometer to ensure reproducible rotation speed.

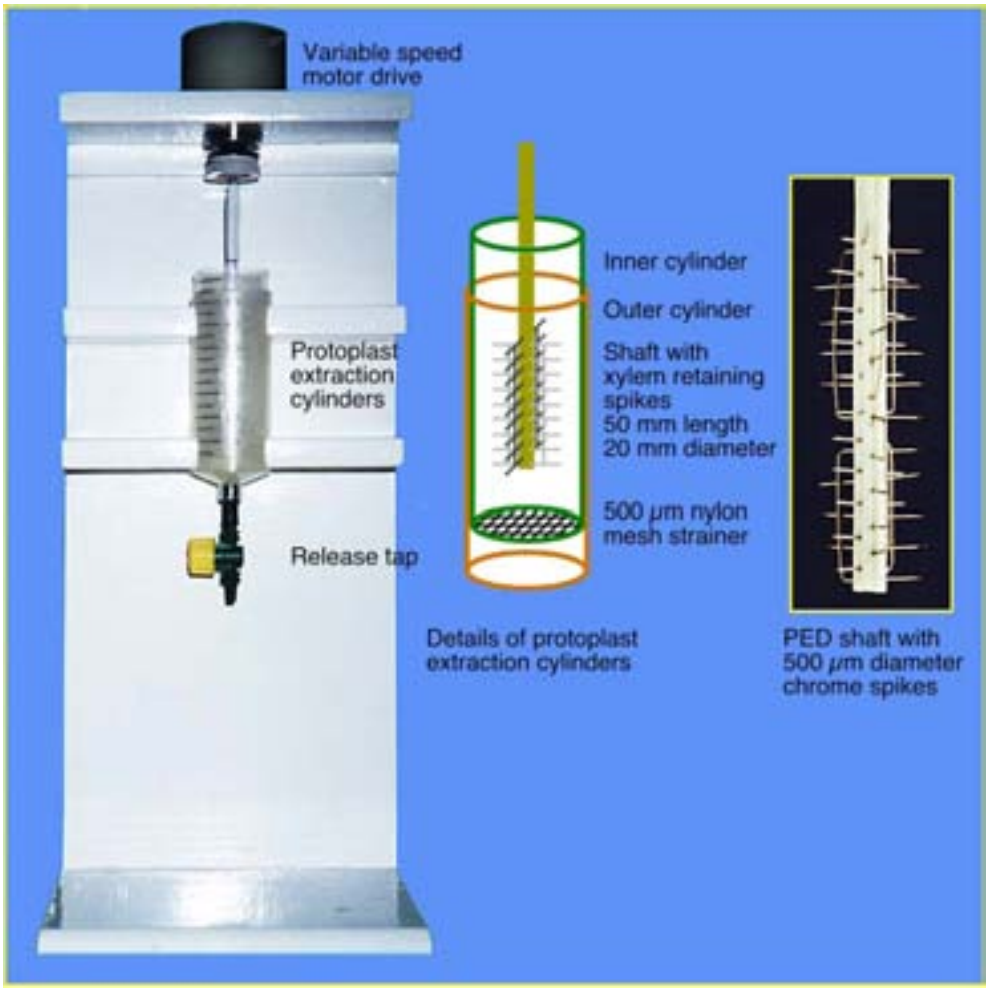

Figure 11 Protoplast extraction device.

Extraction of ray cell stacks

Briefly, the extraction procedure was as follows.

— Developing xylem was scraped, in a sterile manner, from 300 mm stem segments cut from *E. camaldulensis* stems in early spring.

— Developing xylem was wrapped around the shaft of the PED and pre-plasmolysed in increasing concentrations of mannitol (0.3-0.7 M) and sucrose (0.7 M) with the PED rotating at 200 rpm.

— Developing xylem was digested in protoplast extraction solution (PES) pH 5.8 (cellulase 0.7%, pectinase 1.5%, sucrose 0.7 M). Digestion was performed in 2 stages – for 180 minutes at 25^{O}C and 200 rpm, then for 1 minute at 25^{O}C and 500 rpm.

— The release tap was opened and digested material was released into sterile 15 ml Falcon tubes.

— The tubes were centrifuged at 100 x g for 5 minutes.

— The pellet was resuspended in modified MS medium (Murashige & Skoog 1962) and washed 3 times with centrifugation at 100 x g for 5 minutes. This procedure removed fine particulate material.

— The pellet was resuspended in 12 ml of fresh PES in a 15 ml Falcon tube and digested for 30 minutes at 25^{O}C while being shaken on a vertical turntable rotating at 1 rps.

— The suspension was washed as described previously then strained through a 200 m polyester mesh to remove large particulate material.

This final suspension contained large numbers of viable ray cell stacks, no other viable cells and small amounts of amorphous debris.

Molecular pathways involved in tylosis formation

We had originally considered utilising expression of putative eucalypt homologues of animal programmed cell death (PCD) genes as a means to initiate early ray cell death, thereby preventing all aspects of heartwood formation. This was based on the notion that heartwood formation, like other developmental processes in plants, ends in PCD. However, although these genes almost certainly exist, progress in characterising their activity in plant research world-wide has been slow. In the time-frame of the project a more precisely targeted approach seemed more likely to achieve success. Therefore, the emphasis of our research was switched to isolation and utilisation of α-expansin genes. These comprise a large multi-gene family and code for proteins that appear to be essential for the orderly enlargement of cells in all phases of plant development. The highly specific nature of α-expansin activity suggests that transformation of cells with tylosis-specific constructs might provide a means to regulate tylosis formation without affecting development of other cell types. So far neither tylosis-specific genes nor eucalypt-specific α-expansin genes have been reported.

The following account of the role of expansins in plant cell growth is summarised from Cosgrove (1998; 2000a; 2000b) and Cosgrove *et al.* (2002). Turgor pressure triggers plant cells to increase in size and modify their shapes. This initiates cell wall relaxation which in turn allows cells to uptake more water for expansion as new wall material is inserted within existing wall material. These processes are maximised at acid pH, resulting in "acid growth", and are catalysed by proteins called expansins. It is thought that expansins enable cell wall relaxation by disrupting non-covalent bonds between cellulose microfibrils and matrix glucans that adhere to the microfibrils. Expansin proteins were first identified in cucumber hypocotyls. Since then many additional expansins have been identified from many other sources including commercial species of *Populus* and *Pinus*. These proteins comprise the α–expansin family. A related group of proteins the β-expansins, also known as group 1 allergens, has been identified in grass pollen.

Finding α-expansin genes

A series of non-degenerate PCR primers was designed after careful examination of α-expansin gene sequences from fourteen diverse species listed in GenBank. One possible forward primer site and two possible reverse primer sites were found. Six possible primers were designed for each site (Forward primers FP-1A to FP-1F, Reverse primers RP-1A to RP-1F and RP-2A to RP-2F). These provided theoretical fragment lengths of ~490 and ~230 bp. Primers were tested with double-stranded DNA prepared during cDNA library construction to avoid problems of priming to vector sites. Two primer pairs, FP-1E with RP-2B and FP-1E with RP-1A showed strong bands in the expected length range on 1% agarose gel. FP-1E with RP-1A could be used as a nested primer on the PCR product from FP-1E with RP-2B. Primer sequences were:

FP-1E ATGGGAGGAGCTTGTGGTTACGG
RP-1A GAGGAGGGTTGCACCAGCC
RP-2B CTGCCAGTTCTGCCCCCAGTT

Sequencing PCR products

The PCR product from FP-1E with RP-2B was cut from the gel and purified. It was then sequenced on the Beckman-Coulter CEQ 8000 Genetic Analyzer using FP-1E, RP-1A and RP-2B primers. All three primers yielded sequences homologous to α-expansin sequences published in GenBank. (FP-1E over 187 bp). The sequence from the FP-1E primer is shown below.

CGAACATTTGATCACAGCCAGTGGACTACTGACACTCAACACCGGCGTGCCCTTCAAGCAC
CGCCCTGTTCAACGAACGGCCTGAAGCTGCGGGTCCTGCTACAAGGATAAAGTGCAACCAA
CCACCCCAAGTGGTGCCTCCCCGGCAACATCACCGTCACCGCCACCAACTTCTGCCCTCCTA
ACTACGCCCTTTCCAACGACAACGGCGGCTGGTGCAACCCCCCGCTCCAGCACTTCAATATG
GCCGAGCCGGCATTCCTCCAAATCGCCCAATACAAAGCCGGGATCGTCCCCCATCTCCTTCA
GAATGGGTCCCCTGTTGCTGTAAAGCACAATGAGAGCGCCGCTGGCAGTGGTTATTCCCTGT
GAATTTTCCCACCCCGAGGTCCCCCACCCTTTACGCCTTTATCCCCTCCTCCGACATACCCCT
TTGGGGGGTTGGCCCTTGGAGTTTCGGTCCCTGAAATCCGGGTTGGGCCCCCCGGGTGGNGT
CCTGGGGGGACGCACCTGNGTGGGCNACNCTTTTGCGGCGTGGTTTCAGTATTTACCTCGGG
GGGGGTTCNTTCAGNTA

Extraction of full-length α-expansin cDNA's

Several approaches are being trialled (under new funding) to isolate full-length α-expansin cDNA's, including:

— Probing the library with the longer α-expansin gene fragment.

— Probing a larger library sample.

— Using new non-degenerate PCR primers to extract sequences from polyethylene glycol-purified plasmids from our tylosis cDNA library. Primers are:

VFP-FLS (CGGCCGCATAACTTCGTATAGCATAC), forward primer in vector on 5' side of α-expansin sequence,

FP-FLS (GCATTCCTCCAAATCGCCCAATAC), forward primer from α-expansin sequence already obtained, and

RP-FLS (GTATTGGGCGATTTGGAGGAATGC) reverse primer (complement of FP-FLS, and

VRP-FLS (GAGCTCGCTTGGACTCCTGTTGATAG), reverse primer in vector on 3' side of α-expansin sequence, to extract two fragments of the whole α-expansin sequence (Figure 12). VFP-FLS and RP-FLS extract the 5' end of sequence and FP-FLS and VRP-FLS extract the 3' end of sequence. These two sequences will be combined with the sequence already obtained to provide the complete α-expansin cDNA.

In recent post-project work sequencing using the FP-1E primer has identified an α-expansin sequence with homology to α-expansin sequences in other species over 380 bp.

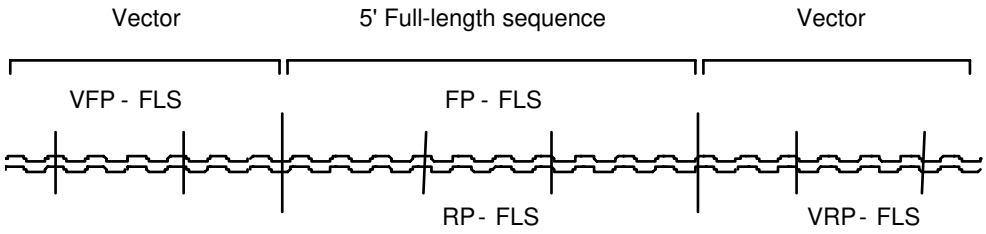

Figure 12 Extraction of full-length α-expansin cDNA

Locating site of action of α-expansin protein

During preparation of outer sapwood of frozen discs for pulverising, care was taken to ensure that cambial tissue and developing xylem was completely removed. In the pulverised tissue the only cells undergoing expansion were ray cells differentiating to tyloses. Demonstrating the expression of a probable α-expansin gene in this tissue provided evidence that this α-expansin gene and/or its promoter was specific to tylosis expansion. To provide further confirmation, we have prepared samples in which extensive tylosis formation was induced (*in vivo* tylosis system) for *in situ* hybridisation. This is a difficult technique, particularly in plant tissue, but if successful will enable us to localise, by microscopy, the timing and expression site of this gene.

Discussion

The fate of ray cells is among the most difficult of forestry research subjects. Molecular pathways involved in extractive synthesis in the transition zone are only now beginning to be understood, while those involved in tylosis formation are completely unknown. Lack of progress is a consequence of the transitory activity of ray cells in the transition zone, their location within the stem and interference from polyphenols. Work in this project has circumvented some of these barriers and provided new tools including, the Xylem Xapper, tylosis and outer sapwood cDNA libraries, and *in vitro* systems. These tools are applicable not only to the study of tylosis formation but also to the study of extractive synthesis and release.

We have sequenced the first ever α-expansin cDNA fragment from a eucalypt species. Confirmation that an α-expansin gene homologue was present in a eucalypt species was not surprising but the fact that this

sequence appears to be associated with tylosis expansion is highly significant and provides a unique opportunity to specifically target tylosis formation. It is now feasible to consider regulation of tylosis initiation using an RNAi (RNA interference) construct based on our α-expansin sequence, although experience in our laboratory suggests this will be a time consuming and difficult procedure. This will be followed by trials in glasshouse trees to confirm that tylosis formation can be prevented *in vivo*.

Communication of results

The Xylem Xapper manuscript has been published. A second manuscript describing earlier work titled "Transpiration-assisted perfusion fixation provides *in situ* preservation of developing ray parenchyma cells in *E. nitens*" has been submitted to Journal of Microscopy. A manuscript titled "A recirculating flow *in vitro* system for the morphological and molecular study of wound-response tyloses in eucalypts" will be submitted for publication when transmission electron microscopy results are available and a manuscript relating to the role of expansins in tylosis formation will be submitted for publication when *in situ* hybridisation results are available.

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