MOLECULAR XYLEM CELL WALL STRUCTURE OF AN INCLINED CYCAS MICRONESICA STEM, A TROPICAL GYMNOSPERM

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SUMMARY

The molecular structure of tracheid walls of an inclined eccentrically grown stem of Cycas micronesica K.D. Hill did not differ between the upper and lower side. The absence the typical molecular features of compression wood tracheids, i.e. an increased galactose and lignin content as well as an increased microfibril angle, indicated that cycads do not have the ability to form even very mild forms of compression wood, which lacks anatomical features commonly observed in compression wood. Analysis of the sugar monomers in Cycas micronesica tracheids did reveal a rather unique composition of the non-cellulosic polysaccharides for a gymnosperm. The low mannose and high xylose content resembled a cell wall matrix common in angiosperms. The crystalline cellulose structure in Cycas micronesica tracheids closely resembled those of secondary cell walls in Picea sitchensis (Bong.) Carr. tracheids. However, the spacing between the sheets of cellulose chains was wider and the cellulose fibrils appeared to form larger aggregates than in Sitka spruce tracheids.

Key words: Cycas micronesica, Cycadaceae, compression wood, cellulose structure, hemicelluloses, phylogeny, tracheid.

INTRODUCTION

Most gymnosperms are known to control their spatial orientation by the formation of compression wood. Compression wood is formed on the lower (compression) side of inclined stems and branches (as summarised by Timell 1986). It exerts an expanding force redirecting the plant orientation. Compression wood shows unique morphological characteristics like intercellular spaces. The tracheids are rounded and thicker walled, lack the S3 layer, and have helical grooves in the S2 layer. On the molecular scale the predominant compression wood features are a high microfibril angle and lignin content as well as the presence of a β-1,4-galactan that is not found in normal wood and is revealed only by microchemical analysis. These characteristics vary in their specificity depending on compression wood severity and species, and are normally accompanied by accelerated radial growth.

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According to Timell (1983) compression wood has existed as a vital tissue in gymnosperms for 300 million years. Coniferales, Taxales and Ginkgoales form compression wood in response to geotropic stimuli (Westing 1965; Timell 1986). For a long time it was assumed that Cycadopsida were unable to react to geotropic stimuli (Westing 1968). However, Fisher and Marler (2006) have recently reported accelerated growth on the lower (compression) side of an inclined Cycas micronesica stem, which was clearly a response to a geotropic stimulus. The anatomy of the tracheids located on the upper (tension) and lower (compression) side did not differ when studied with light microscopy. Compression wood tracheid anatomy has developed during evolution, exhibiting the least difference from normal wood in evolutionarily ancient species like e.g. Ginkgo biloba L. (Timell 1983). As the Cycadopsida having been described as ‘living fossils’ (Chamberlain 1935), only minor anatomical differences between normal and compression wood tracheids might be expected in this group. Recently the presence of β-1,4-galactan has been reported to be a very sensitive indicator of compression wood in Picea sitchensis, revealing compression wood cells that lack any of the typical anatomical features (Altaner et al. 2007).

The formation of compression wood is not necessarily specific to gymnosperms (Höster & Liese 1966). Angiosperms which form tracheids tend to form tissue resembling compression wood (Timell 1983; Baillères et al. 1997), while it is thought that fibre-containing members of the Gnetales form tension wood fibres as do the more highly developed angiosperms (Tomlinson 2003; Tomlinson & Fisher 2005).

Genetic techniques have been used to unravel gymnosperm phylogeny, including the Cycadopsida (Chaw et al. 1997, 2005; Schmidt & Schneider-Poetsch 2002). Analysis of the phytochrome genes placed the Cycadopsida and Ginkgopsida in a sister group to the Pinopsida, while the Gnetopsida were separated earlier (Schmidt & Schneider-Poetsch 2002). The same close relationship between Ginkgopsida and Cycadopsida was found by analysis of rRNA (Chaw et al. 1997; Stefanovic et al. 1998). Other published phylogenies place the Ginkgopsida and Cycadopsida slightly further apart. Alongside genomic techniques, the chemical composition of the cell wall has been shown to yield valuable taxonomic information (Carnachan & Harris 2000; Nothnagel & Nothnagel 2007; Ros et al. 2007).

The cell wall microchemistry of tracheids of an eccentrically thickened, heavily inclined Cycas micronesica stem was investigated. This microchemical data holds valuable information on the phylogeny of gymnosperms and the capability of compression wood formation of cycads. To the best of our knowledge no information on the chemical composition of cycad tracheids has been published before.

**MATERIAL AND METHODS**

Description of the origin and anatomy of the Cycas micronesica samples can be found in Fisher and Marler (2006). The availability of this old tree of an endangered species was opportunistic because the tree was infected with an introduced and lethal Aulacaspis cycad scale insect (Aulacaspis yasumatsui Takagi) (see: http://www.iucn.org/themes/ssc/sgs/csg/pages/CAS.htm). The tree was heavily infected and had no prognosis for
recovery. While we assume that the damage to leaves and roots by the insect was no more than a year old and would have little if any effect on the chemistry of the tracheid walls of old xylem, we have no direct information on this aspect. From samples originally fixed and stored in 70% ethanol two 2-mm-thick radial profiles were cut with a precision twin-bladed circular saw. The cross-sectional strips were used for transmitted light measurements in the wet state. The longitudinal-radial strips were dried and used for microfibril angle measurements by wide angle X-ray scattering. From the remainder, vascular tracheid containing tissue was separated from the parenchymatic tissue for use in solid state NMR and chemical analysis.

_Picea sitchensis_ and _Apium graveolens_ L. samples have been described earlier (Altaner et al. 2006; Kennedy et al. 2007).

**Acid hydrolysis**

Isolated tracheids were disrupted in an Ultra-Turrax homogenizer. Because the parenchymatic tissue tested positive for starch using an Iodine test, the milled samples were boiled for 1h in water and washed several times with hot deionised water in order to remove starch. 200 mg dried material was subjected to a 2-step hydrolysis according to Tappi standard T 249 (Tappi 2000). The sugar monomers were quantified by HPLC ion chromatography. The hydrolysis residue was weighed as an indication of the lignin content.

**Wide angle X-ray scattering**

Wide angle X-ray scattering patterns were obtained on a Rigaku R-AXIS RAPID image-plate single crystal diffractometer with Mo radiation (λ = 0.71073 Å) using a 0.8 mm collimator. Measurements were at ambient temperature and relative humidity. The microfibril angle was calculated according the method proposed by Cave (1966).

**NMR**

The $^{13}$C CPMAS NMR spectra were recorded at 100.56 MHz in a Varian VNMRS spectrometer and referenced to tetramethylsilane. Acquisition time was 40 ms with a recycle time of 1 s. The isolated tracheids (c. 100 mg) were hydrated with ~30% water prior to the measurement.

**RESULTS AND DISCUSSION**

**Cell wall characteristic of Cycas micronesica**

The non-cellulosic cell wall polysaccharides of the _Cycas_ tracheids differed in their composition from conifers. While the mannose content in _Cycas_ was lower, xylose and arabinose were more abundant compared to conifers. The sugar monomer composition was not consistent with the predominance of galactomannans normally observed in conifers (Table 1). The sugar composition of _Cycas micronesica_ was in fact closer to angiosperms which have mannose and xylose contents of 0.9–3.8% and 15.1–24.9%, respectively (as summarised by Fengel & Wegener 1989).

Wood of _Ginkgo biloba_, which has been placed in the same combined Cycadopsida/Ginkgopsida class (Schmidt & Schneider-Poetsch 2002) does contain non-cellulosic
polysaccharides found in wood of conifers (including compression wood) (Jabbar Mian & Timell 1960a, b; Timell 1983). In wood of *Gnetum gnemon* L., a member of the Gnetopsida class, which seems to be the earliest branch in the gymnosperm phylogeny (Schmidt & Schneider-Poetsch 2002), non-cellulosic polysaccharides more similar to angiosperms have been described (Melvin & Stewart 1969). Alkali extracts of primary walls of the cycad *Encephalartos longifolius* seem to contain a higher mannose to xylose ratio than the conifer *Metasequoia glyptostroboides* (nothnagel & nothnagel 2007). Unfortunately, we cannot draw a definite conclusion because these authors did not report yields.

The lignin structure in *Cycas micronesica* tracheids was found to be a guaiacyl lignin typical of conifers. The solid-state $^{13}$C CPMAS NMR spectra were very similar to *Picea sitchensis* wood (Fig. 1). No signals for syringyl units ($\sim$153 ppm), which are typical for guaiacyl-syringyl lignin of angiosperms, were present. This was consistent with earlier reports (Erickson & Miksche 1974; Ros *et al.* 2007), which are based on the wet chemical analysis of lignin isolated from leaf stalks of *Cycas revoluta*. Interestingly a guaiacyl-syringyl lignin that was reported for the cycad *Stangeria eriopus* (Erickson & Miksche 1974) would be in accordance with reports of positive Mäule reactions of Cycadopsida and Gnetopsida (Melvin & Stewart 1969). *Ginkgo biloba*, on the other hand, possesses a hydroxyphenyl-guaiacyl lignin (Erickson & Miksche 1974; Terashima *et al.* 2002; Ros *et al.* 2007). Ester-linked ferulic acid was reported to be present in primary leaf cell walls of gymnosperms including Cycadopsida (Carnachan & Harris 2000).

CPMAS NMR spectra of *Cycas micronesica* tracheids also showed typical acetyl (21 ppm) contents.

Table 1. Chemical composition of *Cycas micronesica* tracheids (lower: compression side; upper: tension side). Weight percentage of released sugar monomers based on dry mass of tracheid cell walls.

<table>
<thead>
<tr>
<th>Position of wood samples</th>
<th>Basal lower</th>
<th>Basal upper</th>
<th>Apical lower</th>
<th>Apical upper</th>
<th><em>Cycas</em> tracheids (mean ± stdev)</th>
<th>Conifers (normal wood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose (%)</td>
<td>3.0</td>
<td>2.3</td>
<td>2.7</td>
<td>2.1</td>
<td>2.5±0.19</td>
<td>1–2.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rhamnose (%)</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3±0.04</td>
<td>&lt;0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Galactose (%)</td>
<td>1.7</td>
<td>1.7</td>
<td>1.8</td>
<td>1.7</td>
<td>1.7±0.02</td>
<td>1–3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose (%)</td>
<td>37.0</td>
<td>41.2</td>
<td>35.0</td>
<td>31.6</td>
<td>36.2±2.00</td>
<td>36.4–47.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Xylose (%)</td>
<td>10.3</td>
<td>12.0</td>
<td>9.9</td>
<td>6.8</td>
<td>9.8±1.08</td>
<td>3.3–7.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mannose (%)</td>
<td>4.4</td>
<td>5.0</td>
<td>4.0</td>
<td>2.5</td>
<td>4.0±0.53</td>
<td>7.4–13.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total sugar (%)</td>
<td>56.7</td>
<td>62.4</td>
<td>53.7</td>
<td>45.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolysis residue (%)</td>
<td>25.7</td>
<td>25.8</td>
<td>25.1</td>
<td>31.2</td>
<td>27.0±1.4</td>
<td></td>
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<tr>
<td>Yield (%)</td>
<td>82.4</td>
<td>88.2</td>
<td>78.8</td>
<td>76.3</td>
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</table>

<sup>a</sup>) As summarised by Timell (1986).

<sup>b</sup>) As summarised by Fengel and Wegener (1989).
Cellulose structure of Cycas micronesica

From the diffraction pattern some conclusions on the supramolecular structure of the cellulose in Cycas tracheids can be drawn. No difference in the cellulose structure in Cycas tracheids was found between the 4 samples originating from different positions in the stem. The lateral 2θ-profile (Fig. 2) was found to have characteristics similar...
to coniferous secondary cell wall cellulose (mature Sitka spruce wood). The [200] reflection, expressing the distance between the sheets of cellulose molecules was at a 2θ angle of 10.21°±0.09 (n=11), closer to wood cellulose (10.32°±0.14 (n = 6)) than to the primary-wall cellulose of celery (*Apium graveolens*) collenchyma (9.91°±0.08 (n = 6)). Similarly the profile of the [110] and [1-10] reflections also was closer to spruce wood than to celery cellulose, for which these two reflections merge into a single peak at 7.5°, indicating a rather rectangular unit cell in transverse section (Müller et al. 2002). The lateral cellulose crystal size in the [200] direction was calculated with the Scherrer equation. The lateral dimensions of the cellulose crystallites were similar in *Cycas* tracheids (2.38 nm ± 0.11 (n = 11)), Sitka spruce tracheids (2.56 nm ± 0.09 (n = 6)) and celery collenchyma primary walls (2.41 nm ± 0.13 (n = 6)).

Solid-state NMR spectroscopy provides not only information on the crystalline cellulose phase but also the non-crystalline part (as summarised by Maunu 2002). The crystalline phase of the cellulose (89 ppm, 65 ppm) appeared to be identical in *Cycas micronesica* and *Picea sitchensis* tracheids (Fig. 1). Differences in the amorphous polysaccharide phase (cellulose and hemicelluloses) were seen. The hemicelluloses resonate at 102, 82 and 62 ppm. The assignment of mannan and xylan signals is still unclear and most likely to be dependent on the fine structure of the polysaccharides themselves (Liitiä et al. 2003). The amorphous C6 (hexoses) and C5 (pentoses) signal at ~62 ppm appeared wider in the spectra of *Cycas* tracheids. Concurrently the hemicellulose C1 signal at 102 ppm and the C4 signal at 82 ppm were more intense in *Cycas* reflecting the higher hemicellulose content (Table 1). Cellulose chains located at accessible and inaccessible microfibril surfaces resonate around 84 ppm (Larsson et al. 1997; Hult et al. 2000). In the *Cycas* sample a wider peak appeared to underlie this region suggesting a higher amount of inaccessible surface chains and/or hemicelluloses. The sharper doublet assigned to accessible fibril surfaces seemed more pronounced in the Sitka spruce sample. Hence the NMR spectra indicated larger cellulose fibril aggregates in *C. micronesica* compared to *P. sitchensis*. An influence of the chemical composition of the cell wall matrix on the cellulose fibril aggregate size has been suggested previously, becoming wider with increasing lignin and possibly hemicellulose content (Donaldson 2007).

**Reaction wood in *Cycas micronesica***

No significant difference in chemical composition between the lower (compression) and upper (tension) side of an inclined stem of *C. micronesica* was found in tracheids at the two sampled stem positions (Table 1). The absence of variation in the galactose content indicated that this cycad species does not have the ability to form compression wood like conifers (as summarised by Timell 1986). This was supported by the constant hydrolysis residue values, which are an indication of the lignin content. The values were around 25% with the exception of the apical upper sample (31%). The samples originating from the more apical part of the stem (especially on the upper side) showed signs of pre-existing degradation (Fisher & Marler 2006). This might be the reason for the slightly different chemical composition of the apical upper sample (lower total sugar content, higher hydrolysis residue – proteins, inorganic matter – lower yield).
The microfibril angle, similarly to the chemical composition, did not vary significantly between tracheids located at the upper or lower side of the inclined *C. micronesica* stem (Table 2). At 11.4°, the microfibril angle values were as low as in mature normal conifer tracheids (as summarised by Timell 1986). The fact that the tracheids did not run in a strictly axial direction complicated the determination of a microfibril angle value in the cycad samples. Therefore the minimum microfibril angle, ranging from 11.4° to 14.5° for the individual samples, is a more representative figure than the mean microfibril angle.

Transmitted light is regarded as a precise and reliable way to identify compression wood in conifers, where compression wood appears more opaque (Pillow 1941). Such differences in opacity were not observed in the vascular tissue between the upper and lower side of the stem (data not shown).

None of the three molecular characteristics (increased galactose and lignin content as well as a higher microfibril angle) of compression wood was found in a heavily inclined, eccentrically grown *Cycas micronesica* stem. This confirms the previously reported anatomical observations (Fisher & Marler 2006) and is in line with earlier experimentally unsupported claims of Westing (1965, 1968) and Timell (1983) that cycads do not form compression wood. However, cycads are able to respond to geotropic stimuli by eccentric growth, as is known from some monocotyledons having secondary growth (Fisher 1975). Geotropic responses have been questioned for Cycadopsida (Westing 1968) and were only reported for roots (Chamberlain 1935). A closer examination of a *Dioon spinulosum* cross section published by Chamberlain (1911) indicates eccentric growth and therefore a possible geotropic response of this cycad species. The soft nature of the stem tissue surrounding the tracheids in cycads is a likely reason for the lack of compression wood tracheids as means of restoring an inclined stem to the vertical. The expansive force created by compression wood tracheids (Boyd 1973) would be difficult to translate into a righting action on the stem. Similar to the situation in Agavaceae, where eccentric growth under the absence of reaction wood cells does not result in a righting of the stem (Fisher 1975), the function of eccentric growth in cycads seems primarily to stabilise the dislocated stem. Reorientation of the growth axis probably occurs in the region of primary growth (Fisher & Marler 2006).

**CONCLUSION**

*Cycas micronesica* tracheids of an inclined stem showed no signs of molecular features (*i.e.* increase microfibril angle as well as galactose and lignin content) commonly as-

Table 2. Microfibril Angle (MfA) in *Cycas micronesica* tracheids (lower: compression side; upper: tension side). Standard deviation in parentheses.

<table>
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<th>Basal lower</th>
<th>Basal upper</th>
<th>Apical lower</th>
<th>Apical upper</th>
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</thead>
<tbody>
<tr>
<td>Mean MfA</td>
<td>18.0 (± 3.6)°</td>
<td>16.6 (± 3.3)°</td>
<td>14.6 (± 2.3)°</td>
<td>19.2 (± 3.7)°</td>
</tr>
<tr>
<td>Minimum MfA</td>
<td>13.7°</td>
<td>12.8°</td>
<td>11.4°</td>
<td>14.5°</td>
</tr>
<tr>
<td>Number of MfA determinations</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>6</td>
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</table>
sociated with compression wood formation. This confirmed the previously published report on the absence of compression wood in this species using light microscope techniques (Fisher & Marler 2006). The non-cellulosic polysaccharides in *Cycas micro-nesica* tracheids are rather unusual in their composition for a gymnosperm and appear more similar to what is known from angiosperms. Information on the molecular structure of the non-cellulosic polysaccharides of evolutionary ancient gymnosperms could allow further insight into the evolution of plant cell walls. Incorporating into such investigations less developed angiosperms, which form compression wood like tissue, could demonstrate whether the chemical and anatomical characteristics of compression wood have indeed evolved as a functional unit.

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