Ion induced changes in the structure of bordered pit membranes

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INTRODUCTION

Self-actuating hybrid materials are used in a wide range of micro-devices ranging from flux control in microfluidic systems using gels (Beebe et al., 2000) to shape memory actuators (Vyawahare et al., 2008). The growing interest in the use of “smart materials” results from the fact that such materials do not require external triggers, rather they simply respond to changes in their surroundings. Such autonomously acting materials occur within the plant vascular network. Trees must move large volumes of water from soil to atmosphere to sustain photosynthetic activity. Because the supply of water in terrestrial ecosystems is often limiting, the ability to tune the distribution of water across plant's dendritic structures is essential to optimize resource use. In this report we use atomic force microscopy (AFM) to investigate structural changes in bordered pit membranes associated with changes in the ionic concentration of the surrounding solution. When submerged in de-ionized water, AFM revealed bordered pit membranes as relatively smooth, soft, and lacking any sharp edges surface, in contrast to pictures from scanning electron microscope (SEM) or AFM performed on air-dry material. Exposure of the bordered pit membranes to 50 mM KCl solution resulted in significant changes in both surface physical properties and elevation features. Specifically, bordered pit membranes became harder and the fiber edges were clearly visible. In addition, the membrane contracted and appeared much rougher due to exposed microfibers. In neither solution was there any evidence of discrete pores through the membrane whose dimensions were altered in response to the ionic composition of the surrounding solution. Instead the variable hydraulic resistance appears to involve changes in both the permeability and the thickness of the pit membrane.

Keywords: bordered pit membrane, xylem, pectin, hydraulic resistance, atomic force microscopy

Ion-mediated changes in xylem hydraulic resistance are hypothesized to result from hydrogel like properties of pectins located in the bordered pit membranes separating adjacent xylem vessels. Although the kinetics of the ion-mediated changes in hydraulic resistance are consistent with the swelling/deswelling behavior of pectins, there is no direct evidence of this activity. In this report we use atomic force microscopy (AFM) to investigate structural changes in bordered pit membranes associated with changes in the ionic concentration of the surrounding solution. When submerged in de-ionized water, AFM revealed bordered pit membranes as relatively smooth, soft, and lacking any sharp edges surface, in contrast to pictures from scanning electron microscope (SEM) or AFM performed on air-dry material. Exposure of the bordered pit membranes to 50 mM KCl solution resulted in significant changes in both surface physical properties and elevation features. Specifically, bordered pit membranes became harder and the fiber edges were clearly visible. In addition, the membrane contracted and appeared much rougher due to exposed microfibers. In neither solution was there any evidence of discrete pores through the membrane whose dimensions were altered in response to the ionic composition of the surrounding solution. Instead the variable hydraulic resistance appears to involve changes in both the permeability and the thickness of the pit membrane.
The obvious next step is to use liquid phase AFM to investigate how the structure of bordered pit membranes changes in response to the ionic composition of surrounding solution and what properties of this composite material are responsible for the changes in its hydraulic properties.

**MATERIALS AND METHODS**

**SAMPLE PREPARATION**

Tobacco plants (*Nicotiana tabacum* L.) were grown in a greenhouse under natural light conditions until stem diameters exceeded 10 mm. A 5-cm-long stem segment was excised and the xylem sectioned longitudinally into 50 μm thick sections, while supplying DI water to prevent samples from drying out. Bordered pit membranes are covered by a secondary wall having 1 by 5 μm elliptical openings. To access and image the pit membrane in AFM, the secondary wall has to be removed without damaging the pit membrane underneath. Longitudinal sectioning sometimes separates from the primary walls leaving intact bordered pit membranes (Figure 1A). As localization of these regions using AFM is exceedingly difficult, the sections were first examined with a 50X optical microscope to locate areas with exposed and undamaged bordered pit membranes. These areas were marked so that they could be located in the AFM. Sections were stored in DI water upon transfer from the light to the AFM microscope. Sample preparation proved to be extremely difficult. Most often the entire process resulted in sections that were unsuitable for AFM scans due to tilt, unexposed or torn bordered pits, problems impossible to detect in light microscope. Thus only a small number of replicates gave useful results despite hundreds of attempts. For visualization purpose only we also used environmental SEM (99% relative humidity in 0.01 MPa pressure) to image exposed bordered pit membranes prepared in the same way as for AFM analysis.

**AFM OBSERVATIONS**

A MFP-3D-BIO™ Atomic Force Microscope (Asylum Research Inc.) was used in the tapping mode to image hydrated bordered pit membranes. Sectioned samples were mounted in the closed fluid cell of the AFM using double-sided tape (3M). Bordered pit samples were submerged and imaged first in DI water followed by imaging in 50 mM KCl (Sigma Aldrich, NE). The cantilever tip was kept in same position to allow for visualization of pit membrane structure resulting from alterations in the ionic concentration of the liquid medium. The sample and AFM cantilever were aligned using an Olympus 1 x 81 inverted microscope (Olympus Inc). A 60 μm long silicon nitride bio-lever (Olympus Inc) provided the necessary flexibility for working with submerged samples. Scan rate was 0.3 Hz, around four to five times slower than with dry samples, with scan speed corresponding to the size of the image. The second harmonic resonance frequency was 51.8 kHz and the image resolution was 512 by 512.

**RESULTS**

**BORDERED PIT MEMBRANE IN DI WATER**

Atomic force microscopy revealed that the hydrated pit membrane is covered with a relatively disorganized material. Surprisingly, distinct microfibrils, which are usually quite obvious in SEM images (Figure 1A), were not observed. Instead, elevation images reveal a bumpy, but relatively featureless surface across the entire pit membrane (Figure 1B). The roughness of the surface was determined using:

\[ R.M.S. = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (y_i - \bar{y})^2}, \]

where \( n \) is the number of analyzed pixels, \( y \) is measured height and \( \bar{y} \) is the height average. Average roughness was 12.6 nm with SD = 2.18 and \( N = 10 \) (\( N \) is the number of independent roughness determinations). The surface of the bordered pits lacked any visible holes or voids, which are often reported from analysis of dry samples.

The relatively featureless structure of the material covering bordered pit membranes and lack of the visible cellulosic fibers found on dry imprints of the membranes in SEM studies raises the question of the physical properties of this surface covering. We believe that the microfibrils are hidden inside this material and that under dry conditions the material collapses, behaving as hydrogel. To determine if this hypothesis was true we adjusted the AFM tip position such that the AFM tip penetrated deeper into the covering material. During the tapping mode, AFM is designed to keep the position from the sample by feedback control. The position from the surface is determined by the set point, which is typically 80% of free amplitude (a delicate tap that results in very low tip displacement). To probe deeper into the pit membrane we reduced this set point to 55%. If the covering of the surface was a hard material, the randomness and relative lack of features on the image should remain similar to what was observed using a set point equal to 80% of free amplitude. Alternatively, the increase of force would result in AFM tip damage (as happen in every case on vessel wall). However, with bordered pits the decrease in free amplitude revealed fiber-like structures under the softer cover (Figure 2). The presence of scratch marks across the image may have resulted from material adhering to the tip being dragged along in the scanning direction.

**BORDERED PIT MEMBRANE IN KCl**

Replacing DI water with a 50 mM KCl solution resulted in an immediate and marked change in membrane structure. We chose...
50 mM KCl based on previous experience that this concentration results in the maximum effect on membrane hydraulic properties (Zwieniecki et al., 2001). Upon exposure to 50 mM KCl, the cantilever tip had to be moved downward around several hundred nanometers to have the same set point. This suggests that the top surface of the bordered pit membrane also moved downward. This drop may indicate the collapse of the soft material. In support of this, the surface was much harder and the microfibrils more exposed (Figure 3). We did not observe the formation of any holes or cracks in the membrane that could have contributed to a lower hydraulic resistance. Instead, the major changes observed included a drop in height which can be interpreted as a decrease in overall membrane thickness (Figure 4) and a significant increase in surface roughness. The average roughness of bordered pit surface increased from 12.6 (in DI water) to 16.6 nm (SD = 1.36 and N = 10; in 50 mM KCl). This change was significant with $t$-value = 4.94, df = 18, and $p < 0.001$. The increase in mean roughness is consistent with a reduction in membrane thickness. We also observed a change in the signal to noise ratio from 4.2 to 5.5 determined as mean to SD. This change in signal to noise ration was consistent with the presence of harder structures, such as microfibrils, covered by a thinner layer of hydrogel.

DISCUSSION
Comparative analysis of pit membrane surface properties in DI water and 50 mM KCl revealed several important and unexpected properties that enhance our understanding of how water moves through this hybrid material. The current paradigm portrays pit membranes as a microfiber network with discrete holes (Kiss et al., 2009). This view is supported by SEM observations of dry material. In contrast our observation of fully hydrated pit membranes did
not reveal any holes, but instead a continuous surface of soft mate-
rial covering fibers with well pronounced elevation features. Upon 
transfer from DI water to 50 mM KCl, this soft surface underwent a 
conformational change in its physical properties from soft to hard 
and from thick to thin, a behavior that is consistent with the prop-
erties of hydrogel (Tanaka, 1981). We believe that this observation 
provides strong support for the notion that bordered pit mem-
branes are composite materials formed from fibrous structure 
submerged in amorphous gel.

This seemingly simple observation has important implications 
for our understanding of the hydraulic properties of bordered 
pit membranes. In particular, it suggests that water does not flow 
through discrete holes or pores, but instead has to pass through 
the soft gel (pectin) matrix. Thus, the physical description of 
water transport across pit membranes in most angiosperms may 
be similar to what occurs in bio-inspired materials that can sus-
tain tension and operate in a similar manner to xylem or leaves 
(Noblin et al., 2008; Wheeler and Stroock, 2008). In particular, 
we may need to change our models for how tension is gener-
ated in leaves, as well as how the embolism spreads in the xylem, 
from ones dominated by surface tension and meniscal failure to 
one that emphasize the osmotic forces that can occur within 
hydrogels.

This study also shifts our understanding of how pit membrane 
hydraulic permeability is altered in response to ionic strength of 
xylem sap. Increase in hydraulic conductance may result from 
changes in membrane thickness (shortening the water path across 
the membrane) and changes in permeability of the charged 
hydrogel (Mattern et al., 2008), while previously proposed changes 
in the dimension of channels might be less influential (Zwiieniecki 
et al., 2001). This finding underscores the role of pit membranes in 
protection from embolism as ionic strength does not necessarily 
decrease the pressure threshold for the air to pass from one vessel 
to another (Cochard et al., 2010). In addition, the observed com-
posite nature of the pit membrane explains their ability to recover 
from mechanical damage (Sperry and Hacke, 2004), as changes 
in gel hydration allow cellulose microfibrils to shift within the 
network and thus prevent the formation of permanent holes or 
cracks. Such healing properties are especially important for plants 
that sustain multiple cycles of embolism and embolism removal 
during drought stress (Sperry and Hacke, 2004). The conforma-
tion response of pit membranes to variation in ions reported here 
suggests that we need to replace the current emphasis on the struc-
tural framework provided by the cellulose microfibrils to flow 
models dominated by the properties of the gel matrix (Wheeler 
and Stroock, 2008).

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