The Relationships between Xylem Safety and Hydraulic Efficiency in the Cupressaceae: The Evolution of Pit Membrane Form and Function

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Water transport in conifers occurs through single-celled tracheids that are connected to one another via intertracheid pit membranes. These membranes have two components: the porous margo, which allows water to pass through the membrane, and the impermeable torus, which functions to isolate gas-filled tracheids. During drought, tracheids can become air-filled and thus hydraulically dysfunctional, a result of air entering through the pit membrane and nucleating cavitation in the water column. What are the hydraulic tradeoffs associated with cavitation resistance at the pit level, and how do they vary within the structural components of the intertracheid pit? To address these questions, we examined pit structure in 15 species of Cupressaceae exhibiting a broad range of cavitation resistances. Across species, cavitation resistance was most closely correlated to the ratio of the torus to pit aperture diameter but did not vary systematically with margo porosity. Furthermore, our data indicate that constraints on pit hydraulic efficiency are shared: the pit aperture limits pit conductivity in more drought-resistant taxa, while increased margo resistance is more likely to control pit conductivity in species that are more vulnerable to cavitation. These results are coupled with additional data concerning pit membrane structure and function and are discussed in the context of the evolutionary biogeography of the Cupressaceae.

Water transport in conifers occurs through narrow, single-celled conduits (tracheids) that are organized in overlapping, longitudinal files. This simple, homoyalous arrangement represents an ancestral vascular design that has remained remarkably consistent since its first appearance in the progynosperms of the Mid-Devonian (Taylor et al., 2009). However, the small size of tracheids can impose a high resistance to water transport as compared with the large, hydraulically efficient vessels present in many angiosperms (Hacke et al., 2004; Sperry et al., 2006). Despite this handicap, conifer tracheids can be just as hydraulically efficient as angiosperm xylem for a given conduit diameter, a result that can be wholly attributed to the distinctive

structure of the conifer intertracheid pit membrane (Pittermann et al., 2005; Sperry et al., 2006).

Because pit membranes also function to limit the spread of air from one conduit to another (cavitation), the physiological consequences of the transport efficiency versus cavitation safety tradeoffs in conifer and angiosperm pit membranes have received considerable attention at the pit and xylem levels, whereby cavitation resistance in north temperate woody plants appears to come at the cost of hydraulic efficiency (Pittermann et al., 2006a, 2006b; Sperry et al., 2006; Choat et al., 2008; Domec et al., 2008; Jansen et al., 2009; Schoonmaker et al., 2010). Previous work has shown that the integrated vascular performance of plants is key to understanding species distributions (Sperry et al., 1994; Brodribb and Hill, 1999; Pockman and Sperry, 2000; Choat et al., 2007), and within this framework, pit membranes have the potential to act as the nexus of the cavitation safety versus transport efficiency compromise. Yet, despite our progress, we are just starting to learn how these tradeoffs play out at the level of the pit membrane, particularly in one as complex as that of conifers. Hence, the goals of this study were to determine whether selection has acted to optimize conifer pit membrane performance in a manner that reflects species cavitation resistance and habitat distribution as well as to examine the role, if any, of evolutionary lineage.
Unlike the homogenous pit membrane of angiosperm vessels, the conifer pit membrane is composed of two distinct regions: a thickened, centrally located torus and a porous margo region that surrounds it (Fig. 1; for study species, see Table I; Hacke et al., 2004; Choat et al., 2008; Choat and Pittermann, 2009). When tracheids are water filled, the pit membrane is centrally located in the pit chamber and water moves from tracheid to tracheid through the margo. Should an air-seeding event (cavitation) occur, causing a tracheid to become air filled, (i.e. embolized), the negative xylem pressure in the water-filled tracheid will act on the air-water interface in the margo pores by deflecting the pit membrane in the direction of the functional tracheid, thereby applying the torus against the pit aperture border (Bailey, 1913; Liese, 1965; Liese and Bauch, 1967; Petty, 1972). This valve action of the membrane can create an effective seal that prevents further spread of air in the xylem. Cavitation is thought to occur when the water potential of the water-filled tracheid becomes negative enough to dislodge the torus from its sealing position, allowing air to enter the conduit. Overall, the structure of the torus-margo pit membrane must optimize what at first glance appear to be conflicting functional requirements: on the one hand, cavitation resistance selects for a combination of large tori and small apertures, but on the other hand, hydraulic efficiency favors porous margos, large apertures, and small tori.

One of the first studies to examine the hydraulic resistance of the conifer pit used a physical model to show that 28% and 44% of pit resistance is explained by the torus and pit border (aperture), respectively, with the remaining 28% of pit resistance residing in the margo (Lancashire and Ennos, 2002). By contrast, computational fluid dynamics suggested that the pit aperture explains only 25% of pit resistance, with 25% to 38% resulting from the margo (Valli et al., 2002). The balance of remaining resistances was attributed to the internal architecture of the pit chamber. Both studies relied on physical or computational models that treated the margo as a homogeneously porous mesh, an approach that may have overestimated or underestimated the margo’s contribution to pit resistance. This is not unexpected, because the margo is an intricate, irregularly porous structure that is difficult to replicate in a model. Compounding this complexity is an additional problem: despite one qualitative survey of pits from 120 gymnosperms (Bauch et al., 1972), very little is actually known about the structural variation of the margo, and even less about how this variation could relate to cavitation resistance.

This uncertainty was broadly quantified by Hacke et al. (2004), who combined empirical data of cavitation resistance from a wide sampling of conifers with a model that treated the margo as a heterogeneous, but organized, mesh composed of pores of varying diameters. The assumption was that the xylem pressure at which membrane aspiration occurred ($P_{asp}$) was directly related to the porosity of the margo. Hence, an increase in the number of margo microfibril “spokes” reduced margo porosity, which increased $P_{asp}$, stabilized the torus, and thus conferred a higher resistance to cavitation. Consequently, reduced margo porosity was associated with greater cavitation resistance.

**Figure 1.** SEM images of intertracheid pit membranes belonging to nine Cupressaceae species (of 15) that represent the broad range of observed cavitation pressures. The opaque torus region of the membrane ($T$) is held centrally by the microfibrils of the margo ($M$). Visually, increased cavitation resistance appears to be associated with increased margo porosity, but quantitative estimates of margo resistance made on the most intact regions of the pit membranes (Fig. 8) revealed no differences among the species surveyed.
Given that conifer tracheids are, on the whole, significantly shorter and narrower than angiosperm vessels, Hacke et al. (2004) recognized that it is essential for the structure of the pit membrane to be optimized for hydraulic efficiency, in addition to the basic requirement of cavitation safety (Hacke et al., 2004). To this end, the models of Hacke et al. (2004) suggested tight scaling between the pit aperture and torus diameter, whereby the torus-aperture overlap was sufficient to achieve a required resistance to cavitation without compromising pit hydraulic efficiency. Specifically, insufficient torus-aperture overlap required a dense margo to achieve a given air-seed pressure, while excessive overlap (due to increased torus diameter and smaller aperture diameter) reduced both the margo area available for water transport and the aperture conductance (Hacke et al., 2004). Interestingly, increased rupture of the margo microfibrils during membrane aspiration was another consequence of excessive overlap, because shorter microfibrils were subject to stretching beyond their inherent tensile strength. Since conifers can experience repeated cycles of cavitation and embolism (Sperry et al., 1994; Mayr et al., 2002), suggesting that the pit membrane can rebound from an aspirated position (Sperry and Tyree, 1990), it seems reasonable to assume that the key constituents of the torus-margo pit membrane have evolved to scale in a manner that optimizes the safety/efficiency tradeoff in light of fixed, biomechanical limitations imposed by the properties of cellulose.

Although the degree of margo variation was unknown to Hacke et al. (2004), they presented important ideas about the functional morphology of conifer pit membranes that have since been confirmed. First, the notion that it is the torus-aperture overlap that determines cavitation safety was recently validated in three species of Pinaceae as well as in stems of Douglas fir (Pseudotsuga menziesii) at different heights (Domec et al., 2008; Hacke and Jansen, 2009). Second, recent studies have shown that the pit aperture controls pit hydraulic conductivity and transport efficiency in the distally located xylem of tall Douglas fir trees as well as across a range of cavitation pressures in different species.

### Table 1. Study species, figure abbreviations (Fig. Abbrevs.), locations (SFBG, San Francisco Botanical Garden, San Francisco; UCBG, University of California Botanical Garden, Berkeley, CA; UCSC, University of California, Santa Cruz, Arboretum, Santa Cruz, CA), and species natural history (Farjon, 2005)

<table>
<thead>
<tr>
<th>Species</th>
<th>Fig. Abbrevs.</th>
<th>Location and Accession</th>
<th>Phenology, Mature Tree Height, Native Elevational Range, and Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Athrotaxis laxifolia</td>
<td>AL</td>
<td>SFBG not cataloged</td>
<td>Evergreen, 10–15 m, 1,000–1,200 m, montane forests, Tasmania</td>
</tr>
<tr>
<td>Callitris rhomboidea</td>
<td>CR</td>
<td>SFBG #1999-0290</td>
<td>Evergreen, 10–15 m, 0–1,250 m, open woodland, Southeast Australia</td>
</tr>
<tr>
<td>Calocedrus decurrens</td>
<td>CD</td>
<td>SFBG #XY-2004</td>
<td>Evergreen, 60 m, 50–2,960 m, mixed conifer forests, Oregon to Baja California Norte</td>
</tr>
<tr>
<td>Cryptomeria japonica</td>
<td>CJ</td>
<td>SFBG not cataloged</td>
<td>Evergreen, 50–60 m, 1–2,050 m, mixed evergreen forests, Japan</td>
</tr>
<tr>
<td>Cupressus forbesii</td>
<td>CF</td>
<td>SFBG #1980-0055</td>
<td>Evergreen, 10 m, 210–1,400 m, chaparral, Baja California Norte</td>
</tr>
<tr>
<td>Fitzroya cupressoides</td>
<td>FC</td>
<td>UCBG #2007.0165</td>
<td>Evergreen, 50–60 m, 4–1,000 m, emergent tree is evergreen rainforest, Chile</td>
</tr>
<tr>
<td>Glyptostrobus pensilis</td>
<td>GP</td>
<td>UCBG #70.0169</td>
<td>Deciduous, 15–25 m, 1–730 m, river floodplains, deltas, Southern China</td>
</tr>
<tr>
<td>Juniperus californica</td>
<td>JC</td>
<td>UCBG #83.0567</td>
<td>Evergreen, 7–10 m, 500–1,400 m, desert scrubland, Southern California to Baja California Norte</td>
</tr>
<tr>
<td>Libocedrus plumosa</td>
<td>LP</td>
<td>UCSC #81.1172</td>
<td>Evergreen, 30–35 m, 1–600 m, lowland mixed angiosperm and conifer rainforests, New Zealand</td>
</tr>
<tr>
<td>Metasequoia glyptostroboides</td>
<td>MGS = stem</td>
<td>UCBG #49.0500</td>
<td>Deciduous, 35–50 m, 750 m, ravines and moist temperate forests, Central China</td>
</tr>
<tr>
<td></td>
<td>MGR = root</td>
<td></td>
<td>Western slopes of Sierra Nevada, California</td>
</tr>
<tr>
<td>Sequoiadendron giganteum</td>
<td>SG</td>
<td>UCBG #2002.1062</td>
<td>Evergreen, over 100 m, 1,400–2,150 m, western slopes of Sierra Nevada, California</td>
</tr>
<tr>
<td>Sequoia sempervirens</td>
<td>SS</td>
<td>Campus, University of California, Berkeley, CA</td>
<td>Evergreen, over 100 m, 1–750 m, moist and foggy climates, Central to Northern California coast</td>
</tr>
<tr>
<td>Taxodium distichum</td>
<td>TD</td>
<td>UCBG #60.1174</td>
<td>Evergreen, 90–95 m, 1,400–2,150 m, mixed conifer montane forests, Eastern California</td>
</tr>
<tr>
<td>Taiwania cryptomerioides</td>
<td>TC</td>
<td>SFBG #1984.93, #1990.616</td>
<td>Evergreen, 60–65 m, 1,750–2,900 m, cool temperate forests, Asia</td>
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<tr>
<td>Widdringtonia cedarbergensis</td>
<td>WC</td>
<td>SFBG #2004-0570</td>
<td>Evergreen, 20–22 m, 1,000–1,500 m, fynbos vegetation, South Africa</td>
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</table>
Douglas fir organs (Domec et al., 2006, 2008). By contrast, estimates of the margo’s contribution to pit resistance have come about by indirect calculations rather than empirical observation. In a survey study that evaluated pit resistance across 19 species of conifers, Pittermann et al. (2006b) concluded that, on average, the pit membrane (torus and margo) probably explains a large fraction of total pit resistance, with less than 10% attributed to the aperture, a major deviation from the results of Hacke et al. (2004) and the other studies cited above. Just how does margo porosity contribute to pit membrane resistance?

In this study, we partitioned the margo and aperture contribution to pit hydraulic resistance by first measuring cavitation resistance in the distal stems and one root belonging to 15 species of Cupressaceae and then combining these data with anatomical measurements obtained via scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of earlywood intertracheid pits. Specifically, we tested the hypothesis that an efficiency-versus-safety tradeoff exists at the pit level and that it is related to the variation in margo structure. We also examined additional features of the pit membrane in order to identify the anatomical characters that combine to affect cavitation resistance and pit hydraulic performance.

The Cupressaceae are an ideal system for exploring the tradeoffs between safety and efficiency in pit membrane structure. They span a wide range of environments, from riparian habitats with ample moisture to desert habitats where precipitation is variable and droughts occur frequently. They are an excellent model for evolutionary studies because their phylogeny is well understood (Gadek et al., 2000) and they have a rich fossil record dating to the Jurassic (Stockey et al., 2005). Conclusions drawn from their anatomy may be all the more relevant as they are the only family of conifers with a worldwide distribution (Farjon, 2005). By sampling broadly across the Cupressaceae phylogeny, we present the functional adaptations of pit membranes across a range of cavitation pressures and can comment on the evolutionary trends of pit morphology in this family.

RESULTS

Species vulnerability to cavitation was determined by measuring the segment percentage loss of conductivity in response to progressively more negative xylem pressures induced by centrifugation (Alder et al., 1997; see “Materials and Methods”). The xylem pressures at which stems showed 50% loss of conductivity (cavitation pressure \( P_{50} \)) spanned a broad range from \(-2.8 \pm 0.62 \) MPa (mean \( \pm \) SD) in the semiriparian Glyptostrobus pensilis to a low of \(-11.3 \pm 3.52 \) MPa recorded in xeric-adapted Widdringtonia cedarbergensis. The \( P_{50} \) of Metasequoia glyptostroboides roots was \(-0.16 \pm 0.06 \) MPa.

SEM images of earlywood intertracheid pit membranes revealed a high degree of variation in membrane structure (Fig. 1). Qualitatively, increasing cavitation resistance was associated with more distinct torus-margo boundaries, thicker tori, torus extensions (Juniperus, Widdringtonia), and the presence of protrusions on the walls of the pit chamber. We also observed that membranes belonging to vulnerable taxa such as Taxodium, Metasequoia (roots especially), and Glyptostrobus were much more fragile and had a tendency to tear and deform when subjected to the electron beam, in contrast to the more cavitation-resistant species, whose membranes remained mostly intact. Although cavitation resistance visually appears to be related to greater margo porosity (compare Athrotaxis with Widdringtonia in Fig. 1), the actual margo structure proved to be so variable that any trends related to cavitation resistance were obscured (see below).

Our measurements of intertracheid pit features on these SEM images revealed no correlation between pit diameter and \( P_{50} \) and only a weak relationship between torus diameter and \( P_{50} \) (Fig. 2, A and B). However, we found that aperture diameter decreased nearly 2-fold with more negative cavitation pressures, ranging from \( 4.5 \pm 0.54 \) \( \mu \)m for \( M. \) glyptostroboides roots to \( 2.3 \pm 0.35 \) \( \mu \)m from stems of \( C. \) forbesii \(( P_{50} = -11.2 \pm 1.78 \) MPa; Fig. 2C).

The ratio of the torus to aperture diameter showed the strongest and most statistically significant relationship with \( P_{50} \) across the 15 species surveyed, suggesting that the sealing function of the torus is essential to controlling air seeding in Cupressaceous conifers (Fig. 3). Specifically, it is the reduction in aperture diameter that determined the extent of torus-aperture overlap, which was calculated according to Hacke et al. (2004) and ranged from 0.01 in Athrotaxis laxifolia to 0.45 in \( W. \) cedarbergensis (data not shown). We calculated an average overlap of 0.19 \pm 0.13, not dissimilar from the 0.24 to 0.3 range predicted to optimize pit conductivity while minimizing air seeding via margo strand rupture (Hacke et al., 2004).

Following standard statistical analysis, the physiological and anatomical data were also analyzed in a phylogenetic context in order to examine whether any of the traits under consideration might have evolved in a correlated manner. In addition, because this study looks at a group of closely related plants, the Cupressaceae, we wanted to test whether phylogenetic relationships within our data either obscured any relationships between traits or, alternatively, led to a false impression of correlation (Felsenstein, 1985; Garland et al., 1992). In order to test for both these effects, we used phylogenetically independent contrasts (PICs; Felsenstein, 1985). This approach has previously been used with hydraulic traits by several authors (Preston and Ackerly, 2003, Maherali et al., 2004; Pratt et al., 2007; Willson et al., 2008). Results from the PIC analysis were largely similar to those from the nonphylogenetic (Tip) analysis, and all relationships between \( P_{50} \) and anatomical data exhibited the same direction whether analyzed as Tips or PICs (Supplemental Figs. S1 and S2; Supplemental Table S1).
Surprisingly, there was little evidence of correlated evolution in any of the pit anatomical characters. However, we found that the key torus-aperture ratio showed the strongest evidence of correlated evolution with $P_{50}$ (Fig. 4). As in Figure 2, there was an indication that this ratio was controlled primarily by changes in aperture ($P = 0.014$, $r^2 = 0.38$) rather than by torus diameter ($P = 0.047$, $r^2 = 0.27$), but other than this, none of the other independent anatomical characters had any correlation coefficient greater than 0.3.

TEM measurements of seven select species that spanned the range of $P_{50}$ values revealed no relationship between margo thickness and cavitation resistance (Figs. 5 and 6), with thickness averaging $194.8 \pm 27.1$ nm. By contrast, the thickness of the torus doubled in response to cavitation resistance, ranging from $288 \pm 215$ nm in *A. laxifolia* ($P_{50} = -3.45$ MPa) to $666 \pm 118$ nm in *W. cedarbergensis* ($P_{50} = -11.3$ MPa). Interestingly, we observed some variation in the electron density of the pit membranes, whereby some tori showed regions of less lignified wall material, such as in *A. laxifolia* and *Taxodium distichum* and the middle lamella of *W. cedarbergensis* (Fig. 5). These qualitative differences in membrane composition, however, could not be functionally linked to a species’ cavitation resistance. Lastly, our TEM analysis revealed that, overall, pit border thickness and aperture depth are weakly correlated with cavitation resistance (Fig. 6), mostly because $P_{50}$, membrane features, and pit border thickness are decoupled in *A. laxifolia*.

Our approach of combined SEM and TEM image analysis with hydraulic methods allowed us to directly test the specific predictions of Hacke et al. (2004) about the structure-function relationships of torus-margo pit membranes. By directly measuring features such as aperture diameter, aperture depth, margo strand length, thickness, and margo porosity, we could identify the hydraulic and biomechanical tradeoffs associated with cavitation resistance across the Cupressaceae. The first of these analyses revealed that pit aperture hydraulic resistance is tightly and significantly correlated with cavitation resistance, decreasing nearly 8-fold over the 10 MPa range of a species’ $P_{50}$ values (Fig. 7A). This is consistent with the observed adjustments in aperture diameter described in Figure 2 and in other studies (Domec et al., 2008). Second, margo hydraulic resistance was shown to be invariable across the spectrum of cavitation pressures, indicating that margo structure is generally unrelated to cavitation resistance in these plants (Fig. 7B). Although...
Figure 4. The phylogeny (left) and phylogenetic independent contrasts (right) of the Cupressaceae species considered in this study. The number on each node corresponds to the independent contrasts data (right) calculated from the $P_{50}$ and torus-aperture ratio values presented in Figure 3.

Figure 5. Transmission electron micrographs of pit membrane cross-sections belonging to six (of seven) selected species of Cupressaceae exhibiting a range of cavitation pressures. A high degree of variation exists in aperture (A) diameter and depth and the thickness of the torus (T), but aside from the torus extension (TE) present in *Widdringtoria cedarbergensis*, margo (M) thickness remained invariable.
the length of the average margo strand (Fig. 9). Consequently, the margo microfibrils experience constant tension across a range of $P_{50}$ values. To look at this from a different perspective, we also calculated the margo strain at aspiration ($e_a$) using pit membrane and torus diameter (Hacke et al., 2004) to show that $e_a$ is, in fact, invariable across $P_{50}$ values (Fig. 9).

DISCUSSION

Our primary objective was to evaluate the pit-level hydraulic tradeoffs associated with cavitation resistance across the Cupressaceae and to specifically address the respective contributions of the margo and pit aperture to total pit hydraulic resistance. Surprisingly, we found no decrease in margo hydraulic efficiency with increasing resistance to cavitation, a result that stems from the high degree of variation apparent in margo strand arrangement in the surveyed taxa (Fig. 7). It seems reasonable to predict that when the membrane is aspirated, a margo composed of thicker strands and/or a denser strand meshwork would stabilize the torus against the aperture and thus prevent air seeding from occurring. Indeed, a denser margo is an important assumption in the modeling work by Hacke et al. (2004) and was, in fact, observed in open-grown, cavitation-resistant individuals of Pinus contorta and Picea glauca relative to those grown in the shade (Schoonmaker et al., 2010). In the same study, shade-grown plants exhibited greater vulnerability to cavitation, a response thought to be a function of the sparse and thin-stranded margo that may have rendered the membrane susceptible to slipping or tearing under negative xylem pressures (Schoonmaker et al., 2010). Given that margo structure in the Cupressaceae appears to be functionally decoupled from $P_{50}$, pit function in the Cupressaceae requires an alternative explanation in addition to the current model, which appears to apply to the Pinaceae.

Recent data suggest that aside from the margo, Pinaceae and Cupressaceae also appear to differ in the structure of the torus, despite showing similar torus-aperture ratios at intermediate $P_{50}$ values (Fig. 3; Hacke and Jansen, 2009). Hacke and Jansen (2009) reported a decrease in torus thickness associated with greater cavitation resistance in three species of Pinaceae, suggesting that thinner, and presumably more flexible, tori may form a tighter seal against the pit aperture during aspiration. By contrast, our results indicate a substantial thickening of the torus in response to more negative $P_{50}$ values (Figs. 5 and 6).

In cavitation-resistant Cupressaceae, once the torus is aspirated and resting against the pit border, the margo could be, up to a point, unnecessary for keeping the torus in place, because the tori of these plants tend to be thick and potentially less susceptible to distortion (Fig. 3). Second, the presence of knobby wall protrusions on the internal side of the pit border may improve the wettability and adhesive properties of the chamber wall (Fig. 1; Kohonen, 2006; Heady et al., 2008; Kohonen and Helland, 2009), so the aspirated torus may actually be quite stable under negative xylem pressures. However, the pit membranes of highly cavitation-resistant species such as Widdringtonia and Juniperus consistently possess torus extensions, which presumably keep the torus from slipping under very negative xylem tensions. Interestingly, Schoonmaker et al. (2010) interpreted the torus extensions in shade-grown Pinaceae differently, regarding them as lesions that increase vulnerability to air seeding by creating a weaker seal between the torus and the aperture.
The presence of thicker and extended tori in cavitaton-resistant Cupressoid and Callitroid clades suggests that in these groups, selection favors pit membranes that minimize air seeding through the seal between the torus and the pit border. In less cavitaton-resistant species such as *T. distichum* and *G. pensils*, the role of the torus in protecting against air seeding is much less clear, because not only is the torus-aperture ratio much lower but the torus itself is thin, poorly differentiated from the margo, vulnerable to deformation as evidenced by its tendency to tear under the SEM electron beam, and often porous, not unlike the homogenous pit membrane of angiosperms (Jansen et al., 2009). It is not unreasonable to suggest that in semiriparian Cupressaceae, air seeding may occur through the minute pores of an aspirated torus or by rupture of the membrane under negative pressure, as suggested by Hacke et al. (2004) and Cochard et al. (2009). In these deciduous conifers, where consistent water availability selects for large pit apertures and high pit transport efficiency (Fig. 7), the margo probably lends little mechanical stability to the aspirated torus.

We were surprised at the high degree of variation in margo resistance ($R_m$), but our measurements of margo porosity were consistently made on the most intact regions of the margo across all species, so our estimates of $R_m$ are conservative. The variation in $R_m$ arises from the presence of one or several large pores, which can have a large impact on $R_m$, because the hydraulic conductance of a pore ($D$) is a function of $D^3$ (see Eq. 3 below). Yet, despite this variation, it is not unreasonable to conclude that in the Cupressaceae, the margo’s contribution to overall pit hydraulic resistance progressively increases in less cavitaton-resistant taxa (Fig. 8). Although it is difficult to identify a precise $P_{50}$ at which margo resistance exceeds aperture resistance, our data suggest that the margo may constrain pit transport efficiency in more mesic-riparian Cupressaceae, or in parts of the tree such as the trunk, roots, or latewood that are typically less resistant to cavitation (Domec et al., 2006). Generally speaking, margo resistance may be higher than aperture resistance in Cupressaceae with $P_{50}$ values that are less negative than about $-5.5$ MPa or that have apertures greater than $4\ \mu$m in diameter. This increased role of the margo in less cavitaton-resistant Cupressaceae is a direct consequence of a concurrent decrease in pit aperture resistance rather than a margo-level trend associated with $P_{50}$.

The idea that the pit aperture controls most of the pit hydraulic efficiency was first proposed by Lancashire and Ennos (2002) using a physical model and later

![Figure 7](image.png)

**Figure 7.** Pit aperture resistance (A), margo resistance (B), and total pit resistance (C) plotted as a function of cavitation pressure. The aperture resistance is calculated on the basis of two apertures per pit.

![Figure 8](image.png)

**Figure 8.** The percentage contribution of the margo to the total resistance of the torus-margo pit membrane increases in taxa that are more vulnerable to cavitation (see text for details).
developed by Hacke et al. (2004), who showed that aperture resistance was consistently higher than the modeled margo resistance in pits of north temperate conifers. Their work suggested that a reduction in aperture diameter was responsible for a 1.7-fold increase in pit resistance across a range of cavitation pressures (Hacke et al., 2004). The similar conclusion of Domec et al. (2008) that the aperture controls pit hydraulic efficiency across a range of \( P_{50} \) values in Douglas fir echoed what seems an obvious and fundamental pit-level hydraulic tradeoff associated with cavitation resistance in conifers. At first glance, our data also support these findings, but closer inspection with SEM suggests that the underlying assumptions of what controls pit hydraulic resistance in the Cupressaceae are more complex, probably because of developmental limitations on the control of margo porosity. Manipulative experiments suggest that margo porosity may be more tightly regulated in the Pinaceae (Schoonmaker et al., 2010), but the degree of pit membrane plasticity in the Cupressaceae is unknown.

Consistent with previous work, our data show that vulnerability to cavitation is strongly controlled by the ratio of torus to aperture diameter, that is, the degree of torus-aperture overlap (Fig. 3; Domec et al., 2008; Hacke and Jansen, 2009). While torus diameter changes modestly across \( P_{50} \), it is the variation in aperture diameter that sets the torus-aperture overlap and ultimately determines the air-seeding resistance of torus-margo pit membranes. The observed increase in torus thickness is consistent with the need for the membrane to structurally withstand more negative xylem pressures in xeric-adapted taxa such as Widdringtonia and Juniperus. A similar correlation between pit membrane thickness and vulnerability to air seeding was recently found for angiosperms (Jansen et al., 2009). By contrast, Hacke and Jansen (2009) observed that thinner tori were associated with an increase in cavitation resistance in three species of Pinaceae, so the phylogenetic influence on the structure and function of conifer pit membranes needs to be broadly examined.

Although our survey of pit membranes in the Cupressaceae is by no means exhaustive, we can comment on the general evolutionary trends in pit structure and function across members of this family. Broadly speaking, the higher cavitation resistance of the Cupressoid and Callitroid grades is a derived character, reflected in part by the trend toward higher torus-aperture ratios, thicker tori, and a progressively more targeted digestion and distinct separation of the torus and the margo (Fig. 1). More quantitatively, the PIC analyses of the cavitation pressure and torus-aperture ratio give us some capacity to trace the evolution of drought resistance in this family. In particular, drought tolerance would most likely have been selected for as the Callitroid and Cupressoid grades of the Cupressaceae radiated into drier habitats of the Paleogene from the semiriparian habitats of their Cretaceous-era Sequoioid and Taxodioid ancestors, as suggested by the fossil record (Florin, 1963; Farjon, 2005; Stockey et al., 2005; see also Willis and McElwain, 2002). Indeed, one of the largest \( P_{50} \) divergences between sister clades is found at the divergence between

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**Figure 9.** A, The maximum tension calculated from margo strand length and torus displacement indicates that individual margo strand is invariable across a range of cavitation pressures. B, Presumably, this is because the average length of the margo strands scales with the mean maximum displacement of the pit membrane during aspiration. C, Calculations of margo strain at aspiration based on measurements of membrane and torus diameter suggest that deformation of the margo is decoupled from the cavitation pressure.
the Taxodioid clade and the Cupressoid and Callitroid clades at node 21. The PIC results also suggest that drought tolerance has been separately derived in the Cupressoid and Callitroid clades, with the largest differences in P50 and the torus-aperture ratio occurring at the split between arid and mesic taxa, such as in nodes 26 and 27 (two of the largest contrasts) of the Callitroids and node 28 of the Cupressoids (Fig. 4). By comparison, the differences between the riparian taxa (Taxodioid clade) and their mesic relatives (Sequoioïd clade) are smaller, as indicated by smaller P50 contrasts at nodes 22 and 19, respectively. It seems likely that the greater vulnerability to cavitation found in the basal Cupressaceae is associated with the high water availability in the mesic-riparian habitats in which these taxa are often found and that this is the ancestral condition for the entire family. Consistent with this, the deciduous Glyptostrobus, Taxodium, and Metasequoia, which grow in semipariparian regions, tend to exhibit thin, fragile, and seemingly diaphanous pit membranes that appear poorly equipped to withstand low water potentials. Thus, both anatomical and independent contrast results support the conclusion that low cavitation resistance is the ancestral condition for the Cupressaceae and that drought resistance is a derived character that evolved separately in the Callitroid and Cupressoid clades. Future studies linking additional xylem-level traits with ancestral state reconstructions may add to our understanding of the evolutionary trajectory of the Cupressaceae.

Lastly, we observed no biomechanical tradeoffs associated with cavitation resistance and the tension imposed on the margo strands of the pit membrane (Fig. 9). The forces imposed upon the margo during aspiration remain invariable because the length of the strands (l) scales with the membrane displacement distance (y) and torus diameter, such that the ratio of l to y remains constant. Our data suggest that, structurally, cavitation-resistant Cupressaceae pit membranes can be classified as type 1 pits, which are for the most part strong, flexible, and resistant to rupture upon aspiration (Hacke et al., 2004). Whether or not “stretch seeding” consistently occurs (i.e. air seeding via stretching of the margo and displacement of the torus) is debatable, because more cavitation-vulnerable taxa such as Taxodium and Glyptostrobus possess pit membranes that are rather fragile, so capillary failure may not be limited to the torus-pit border interface (Cochard et al., 2009). The scaling between pit features is also consistent with the observed proportions between the P50 and the conduit diameter-to-wall thickness ratio (Hacke et al., 2001; Pittermann et al., 2006a, 2006b), aperture and torus diameter, as well as aperture depth and pit border thickness (Fig. 5). This lends support to the idea that, in gymnosperms, where transport is limited to uncellular tracheids, selection has acted to optimize pit function to fulfill both safety and transport requirements (Hacke et al., 2004; Domec et al., 2006, 2008; Sperry et al., 2006).

Where are we with regard to linking pit structure with cavitation resistance across woody plants? The structural heterogeneity of the torus-margo pit makes it an attractive feature to study because theoretical predictions can be reasonably tested using SEM and hydraulic methods, so key traits across all torus-margo pit membranes as well as important differences at the level of the family or genus can be identified among extant conifers. Second, if we are to understand the adaption response of cavitation resistance, it is important to also consider phenotypic plasticity via strategically manipulative experiments such as that of Schoonmaker et al. (2010). For example, our study was conducted on common garden plants grown in a mild climate, but we expect that some species grown in their native environment may have dramatically different P50 values (i.e. Callitris rhomboidea; Brdribb and Cochard, 2009), and this may be reflected in pit structure. Lastly, it is becoming apparent that the process of air seeding may be, in some sense, convergent in both angiosperms and conifers and dependent in part on the thickness of the pit membrane. Jansen et al. (2009) show a tight relationship between air seeding, membrane thickness, and porosity in the homogenous pit membranes of angiosperms that conforms to the predictions made by Sperry and Hacke (2004). Our observations of thin membranes with poorly distinguished tori in deciduous, riparian Cupressaceae suggest that similar patterns may hold in these plants. In this regard, advancing our understanding of homogenous pit membranes, especially the safety/efficiency tradeoffs with respect to cavitation, will fill in an important gap in our understanding of the adaptive nature of interconduit pit membranes in general.

This project focused on the structure/function tradeoffs in pit-level traits, but it is clear that cavitation resistance encompasses a suite of well-studied developmentally coordinated functional traits such as conduit wall thickness, conduit allometry, wood density, and overall physiology (Hacke et al., 2001, 2004; Santiago et al., 2004; Pittermann et al., 2006a, 2006b; Sperry et al., 2006; Jacobsen et al., 2008; Schoonmaker et al., 2010), all of which combine to determine the safety/efficiency tradeoff. Functionally, this means that cavitation-resistant pits cannot be associated with large, thin-walled tracheids that are hydraulically efficient and vulnerable to implosion under tension. A manifestation of this scenario would require that species with efficient xylem such as T. distichum possess pit membranes with narrow apertures, resulting in a combination of traits that would significantly diminish any hydraulic gains associated with large tracheids (Hacke et al., 2004; Sperry et al., 2006). Future work incorporating both the fine structure of pit membranes and tissue-level traits will be required to fully unravel the complex relationships that underlie adaptive variation in cavitation resistance and hydraulic efficiency at the whole plant level.
MATERIALS AND METHODS

Plant Material

All Cupressaceae species were collected from arboreta located on the central California coast, so all plants were subject to similar climatic conditions (Supplemental Table S1). Stem samples from a total of 15 species were collected from Strybing Arboretum in San Francisco’s Golden Gate Park (37.78’N 122.43’W) and from the nearby University of California’s Botanical Garden in Berkeley. Libocedrus plumosa was sampled at the University of California’s arboretum in Santa Cruz (36.97’N 122.03’W). In order to examine the interrelatedness of purely riparian conifer xylem, we collected submerged root material from Metasequoia glyptostroboides trees growing creekside at the University of California’s Botanical Garden in San Francisco. In general, the climate of these three sites is described as Mediterranean, with mild temperatures, dry winters, and abundant summer fog input, and very rare freezing events (mean annual precipitation is 500–580 mm, and mean annual temperature is 15°C–17°C). However, species’ water regimes reflected their natural history, such that individuals of Taxodium distichum were typically found near ponds, while seric-adapted taxa such as Widdringtonia cedarbergensis grew in gardens that were less frequently watered. All species were clearly labeled, associated with accession numbers, and their age and planting history were well catalogued.

Juvenile stems ranging from 5 to 8 mm in diameter were collected from sun-exposed, vigorous regions of mature tree canopies, from heights of 1.5 to 4 m. Five to eight branches were clipped from two to six individuals (depending on permission) and were immediately wrapped in a plastic sac with a wet paper towel until further processing for hydraulic measurements in the laboratory. With the exception of the deciduous conifers (depending on permission) and were immediately wrapped in a plastic sac, where the latter method often reduces hydraulic conductivity (Mayr et al., 2002; Pittermann and Sperry, 2003; Willson et al., 2008).

Hydraulic Measurements

Sampled stems were recut under water to a length of 142 mm, and the distal ends were shaved smooth with a razor blade. Although native embolism is typically less than 10% in well-watered conifer stems, air in the xylem was removed by submerging the stems in distilled and filtered water (0.22 μm; E-Pure filtration system; Barnstead International) and degassing them overnight under “house” vacuum. Stems were degassed rather than flushed because the latter method often reduces hydraulic conductivity (Mayr et al., 2002; Pittermann and Sperry, 2003; Willson et al., 2008).

Hydraulic conductivity (k) was measured according to the method of Sperry (1993) and calculated as the flow rate for a given pressure gradient per unit of stem length. The segments were mounted on a tubing apparatus where k was measured gravimetrically under a pressure of 5 to 6 kPa using filtered water. The flow rate through the segments was determined without a pressure head before and after each gravimetric flow measurement. These background flows were averaged and subtracted from the flow measured in order to improve accuracy.

We used the centrifuge method to determine species vulnerability to cavitation in response to a range of xylem pressures (Pockman et al., 1995; Alder et al., 1997). Stems were secured in a custom rotor designed to fit a Sorvall RC-SC centrifuge and spun for 3 min at speeds that induce a known xylem pressure (Pc). The percentage loss of conductivity (PLC) caused by cavitation in response to a range of xylem pressures (Pc) was calculated from the k measured after spinning, relative to the maximum conductivity (kmax) at Pc = 0 MPa, such that

\[ PLC = 100 \times \left(1 - k/k_{\text{max}}\right) \]  

where \( k_{\text{max}} \) was determined at \( P_c = 0 \) MPa following degassing. The segments were spun to progressively more negative \( P_c \) until the PLC exceeded 90% or, alternatively, until \( P_c = -10 \) MPa, which is the most negative \( P_c \) that can be achieved using the centrifuge. Five to six segments were used to generate the same number of vulnerability curves, each of which was subject to the curve-fitting routine of Pammenter and Vander Willigen (1998). The xylem pressure at which segments exhibited a 50% loss of conductivity (P50) was computed as average ± 1 SD per species.

SEM

Wood samples for SEM were collected from the same individuals and similar canopy locations as described above. Four stem segments that were

5 to 8 mm in diameter and 10 to 15 cm in length were collected from each species, wrapped in a wet paper towel, and transported to the laboratory, where they were recut under water to a 5-cm length and placed in a −10°C freezer for 2 d. These samples were then freeze-dried overnight.

Freeze-dried twig samples were split in half and mounted on aluminum stubs using nail polish to increase conductivity between the sample and stub. Samples were coated with gold-palladium for 4 min at 20 mA using a sputter coater (Leybold). All samples were observed with a field emission electron microscope (Zeiss UltraPlus Analytical) with an accelerating voltage of 3 kV. A minimum of 15 to 20 earlywood intertracheid pit membranes were photographed for analysis.

TEM

Wood samples for TEM were collected from similar canopy locations from the following species: Ailanthus altissima, Callicarpa rhomboidea, Cryptomeria japonica, Cupressus forbesii, Sequoia sempervirens, T. distichum, and W. cedarbergensis. Stem samples measuring approximately 5 to 8 mm in diameter and 5 cm in length were clipped (two stems per species), wrapped in moist paper towels, and immediately mailed by overnight courier to the Jodrell Laboratory at the Royal Botanic Gardens in Kew. Due to customs inspection, the samples were in transit for 3 d.

At Kew, one specimen per species was prepared for TEM. Wood from the last two growth rings was cut into thin longitudinal slivers, cut into 3-mm blocks, and fixed overnight in Karnovsky’s fixative at room temperature (Karnovsky, 1965). After washing in 0.05 M phosphate buffer, the specimens were postfixed in 1% buffered osmium tetroxide for 4 h at room temperature, washed again, and dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, 100%). The ethanol was gradually replaced with LR White resin (London Resin Co.) over several days, with the resin being changed approximately every 12 h. The resin was polymerized in a Gallenkamp vacuum oven at 60°C and 1,000 mbar for 24 h. Embedded samples were trimmed with a Leica EM Specimen Trimmer (Leica Microsystems) and sectioned on an ultramicrotome (Ultracut; Reichert-Jung). Transverse sections of earlywood conduits about 1 and 2 μm thick were cut with a glass knife, heat fixed to glass slides, stained with 0.5% toluidine blue-O in 0.1 M phosphate buffer, and mounted in DPX (containing diisobutyl phthalate, and xylene; Agar Scientific). Ultrathin sections were cut between 60 and 90 nm using a diamond knife. These sections were attached to Formvar (Agar Scientific) and copper grids (100 mesh) and stained with uranyl acetate and lead citrate using a Leica EM Stain Uльтrostainer (Leica Microsystems). Observations were carried out using a JEOL JEM-1210 transmission electron microscope at 80 kV accelerating voltage, and digital images were taken using a MegaView III camera (Soft Imaging System). Measurements were made on at least 15 pit membranes per species.

Phylogenetic Reconstruction

Sequences for two markers (rbcL and MatK) from Gadek et al. (2000) were downloaded from GenBank. Sequences were aligned using ClustalW (Thompson et al., 1994; EMBL-EBI, 2009). Mesquite (Maddison and Maddison, 2009) was used to touch up alignments and concatenate both genes into a matrix, which was then analyzed using maximum parsimony in PAUP* beta 11 (Swofford, 1998), with a random addition sequence and tree bisection and reconnection branch swapping. We were unable to recover the topology found by Gadek et al. (2000) using the smaller number of taxa included in this physiological study. The data were reanalyzed using a constraint tree based on that presented by Gadek et al. (2000), resulting in a single phylogenetic topology with molecular branch lengths. This topology was used for the subsequent comparative analysis.

Comparative Phylogenetic Analysis

The phylogeny was imported into the R statistical environment (R Development Core Team, 2008) using the APE package (Paradis et al., 2004) and used to analyze the physiological data presented in this paper using PICs (Felsenstein, 1985). A single mean measurement for each genus and each trait was used in the PIC analysis, with the exception that only the branch data and not the root data from M. glyptostroboides was used. PIC analysis assumes a Brownian model of branch length evolution. Diagnostic tests (from the Picante package; Kembel et al., 2010) showed that the molecular branch lengths resulting from the combined PAUP* analysis did not obey this assumption.
Estimates of Pit Hydraulic Resistance and Margo Strand Tension

In order to quantify the hydraulic tradeoffs associated with cavitation resistance at the pit level, we calculated the aperture, margo, and pit resistances using methods described by Sperry and Hacke (2004) and Hacke et al. (2004). The hydraulic resistance of one pit aperture \( R_p \) was calculated according to:

\[
R_p = \frac{128t_p v/(\pi D_p^4) + 24v/D_p^2}{l}
\]

where \( t_p \) is the pit aperture depth, \( v \) is the viscosity of water (0.001 Pa s at 20°C), and \( D_p \) is the aperture diameter. For taxa where \( t_p \) could not be obtained from TEM images, \( t_p \) was assigned the average value of 0.7 μm.

The margo resistance \( R_m \) was computed according to Sperry and Hacke (2004), whereby

\[
R_m = \left( \frac{24v}{nD_s^2} \right) f(h)
\]

where \( n \) is the number of pores in the membrane, \( D_s \) is the hydraulic diameter for all the margo pores measured (assuming an equivalent circle diameter of each of the pores), and \( f(h) \) is the proportion of the margo occupied by pores. SEM images were used to estimate margo porosity, and pore areas were measured within an intact, nonappressed section of the margo from an average of four pits using image-analysis software. An average of 350 pores was measured on each photograph. Total pit area-specific resistance was calculated as the sum of \( R_m \) and \( 2 \times R_p \). where \( 2 \times R_p \) accounts for the two apertures present in one pit.

We applied the equation of Petty (1972) to calculate the maximum tension experienced by a single margo strand when the membrane is fully appressed against the pit border. For one radial strand, the tension \( T \) is estimated according to

\[
T = EA \left( \left( f + y \right)^{0.5} / l - 1 \right)
\]

where \( E \) is the elastic modulus of the margo strand (estimated at 5 GPa from Hacke et al. [2004]), \( A \) is the average strand cross-sectional area (2.987 \( \times \) 10^-10 m²) based on the average margo strand thickness (195 nm), \( l \) is the length of the margo strand, and \( y \) is the displacement of the torus against walls of the pit border. Displacement was measured according to Petty (1972) as the distance between the unaspirated, neutrally placed pit membrane and the pit border location where the torus pressures against the pit border during aspiration (approximately the center of the pit border). Margo spoke strain at aspiration \( (e_s) \) was calculated as

\[
e_s = 0.03D_m/(D_m - D_h)
\]

where \( D_m \) and \( D_h \) represent membrane and torus diameter, respectively (Hacke et al., 2004).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF127246 to AF127243, L12537, L12538, L12569 to L12573, L12575 to L12578, L12580, L13172, L25750, L25751, L25754 to L25757, S75127, and X63660.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. A matrix of hydraulic and SEM-based anatomical trait relationships analyzed using PICs.

Supplemental Figure S2. A matrix of hydraulic and TEM-based anatomical trait relationships analyzed using PICs.

Supplemental Table S1. Comparison of standard statistical analysis (Tips) and PICs.

Supplemental Table S2. GenBank accessions used in the phylogenetic reconstructions.

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