Methods
Automated analysis of three-dimensional xylem networks using high-resolution computed tomography

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Summary
• Connections between xylem vessels represent important links in the vascular network, but the complexity of three-dimensional (3D) organization has been difficult to access.
• This study describes the development of a custom software package called TANAX (Tomography-derived Automated Network Analysis of Xylem) that automatically extracts vessel dimensions and the distribution of intervessel connections from high-resolution computed tomography scans of grapevine (Vitis vinifera) stems, although the method could be applied to other species.
• Manual and automated analyses of vessel networks yielded similar results, with the automated method generating orders of magnitude more data in a fraction of the time. In 4.5-mm-long internode sections, all vessels and all intervessel connections among 115 vessels were located, and the connections were analyzed for their radial distribution, orientation, and predicted shared wall area. Intervessel connections were more frequent in lateral than in dorsal/ventral zones.
• The TANAX-reconstructed network, in combination with commercial software, was used to visualize vessel networks in 3D. The 3D volume renderings of vessel networks were freely rotated for observation from any angle, and the 4.5 μm virtual serial sections were capable of being viewed in any plane, revealing aspects of vessel organization not possible with traditional serial sections.

Introduction
Xylem vessels are the primary conduits that distribute water and mineral nutrients in flowering plants. Their low axial resistance allows long-distance transport from roots to leaves and fruit, with intervessel connections completing the integrated network that is also capable of radial and tangential transport. The physical separation of vessels, fixed vessel lengths, and network connections bound by pit membranes act as safety mechanisms that limit the spread of embolisms and xylem-dwelling pathogens while maintaining efficient water transport. However, little is known about how the three-dimensional (3D) organization of xylem networks influences plant hydraulics (Tyree & Zimmermann, 2002), and few studies have measured the spatial distribution, orientation, or frequency of connections. This knowledge gap is the result of difficulties in visualizing the complex, 3D organization of xylem.

Vessel network reconstructions have been produced by analyzing transverse serial light micrographs taken axially through a stem (Braun, 1959; Dimond, 1966). This method is labor-intensive and, consequently, not frequently employed (Huggett & Tomlinson, 2010). The network diagrams that do exist are represented only in two dimensions, often lack a quantitative analysis of network organization, and are typically focused on primary xylem, vascular...
bundles, and phyllotaxy (Larson, 1975, 1980). Secondary xylem reconstructions have revealed the radial and tangential paths of vessels (Bosshard & Kucera, 1973; Tyree & Zimmermann, 2002; Kitin et al., 2004), and some connectivity between growth rings via intervessel pits (Fujii et al., 2001). Zimmermann & Tomlinson (1966) developed an efficient method to photograph and visualize axial serial sections as sequential ‘frames’ in a film, the cinematographic optical shuttle, but reconstruction of vessel networks is still performed manually using transverse serial sections (Gerrath et al., 2001; Kitin et al., 2004).

High-resolution X-ray computed tomography (HRCT) is a diagnostic imaging technique with a micrometer-range resolution that provides continuous serial sections through plant tissue in any orientation. HRCT imaging is based on the same principles as medical CT systems, but a high-intensity, focused X-ray source results in decreased section thickness and image acquisition time. Both two-dimensional (2D) and 3D HRCT have been employed in the cursory analysis of plant-related materials, including the soil and root interface (Aylmore, 1993; Heeraman et al., 1997), fruit development (Verboven et al., 2008), paleobotanical anatomy (DeVore et al., 2006), and the anatomy of wood and vascular tissue (Stuppy et al., 2003; Maeda & Miyake, 2009). Fromm et al. (2001) and Steppe et al. (2004) successfully used HRCT to study wood density; however, the image resolution was insufficient for analyzing intervessel connections. Recent advances in synchrotron HRCT technology have improved image resolution and signal to noise ratio sufficiently so that vessel networks can be visualized to explore the implications of network organization. The analytical potential of HRCT in exploring xylem organization is even greater than its 3D visualization capabilities, as intervessel connections can be assigned 3D coordinates that can be exported and used in model simulations.

Here we describe the use of HRCT to visualize intervessel connectivity in 3D, and present an automated computational method for vessel network analysis in grapevine (Vitis vinifera). Grapevine is used to study plant water relations because of its long, wide vessels (Zimmermann & Jeje, 1981), susceptibility to xylem-dwelling pathogens (Thorne et al., 2006), and both seasonal and drought-induced embolism (Sperry et al., 1987; Choat et al., 2010). Primary xylem organization in the stem related to its distichous phyllotaxy has been well studied (Fournioux & Bessis, 1977; Gerrath et al., 2001), revealing putative distinctions between lateral zones and dorsal and ventral zones of the stem (Stevenson et al., 2004). Xylem organization has also been found to play an important role in the refilling of embolized vessels (Holbrook et al., 2001; Brodersen et al., 2010). Analyzing whole vessel networks will allow us to better understand how the distribution of intervessel connections influences hydraulic conductivity, the movement of pathogens and embolisms, and the ability to adapt or acclimate to a changing environment. We can then expand on previous network modeling efforts (Dimond, 1966; Loepe et al., 2007), and produce improved models of network connectivity.

Materials and Methods

Sample preparation
Grapevines (V. vinifera L. ‘Chardonnay’) were grown from grafted cuttings in 7.6 l pots filled with one-third peat, one-third sand, and one-third redwood compost, with 2.4 kg m⁻² dolomite lime in a glasshouse with supplemental lighting on a 16:8 h light:dark cycle, and drip-irrigated three times daily with Hoagland solution. For dry stem analysis, internode sections from current year stems were excised and dehydrated in a drying oven at 70°C for 48 h. A silicone resin (Rhodorsil RTV 141; Bluestar Silicones Corp., East Brunswick, NJ, USA) was injected via a microcapillary tube into a single vessel following the methods described by Choat et al. (2006). This resin-filled vessel acted as a fiduciary marker for orientation purposes in future dissection and HRCT imaging experiments. The stem samples were then mounted and scanned with HRCT. In a separate experiment, a 10% (w/v deionized water) potassium iodide (KI) solution was injected into a single vessel to determine the utility of this solution as a contrast agent. In addition, fresh internode sections of current year stems were cut from glasshouse-grown plants 24 h before scanning, flushed with deionized water, wrapped in Parafilm, and stored at 7°C for 48 h. A silicone resin (Rhodorsil RTV 141; Bluestar Silicones Corp., East Brunswick, NJ, USA) was injected via a microcapillary tube into a single vessel following the methods described by Choat et al. (2006). This resin-filled vessel acted as a fiduciary marker for orientation purposes in future dissection and HRCT imaging experiments. The stem samples were then mounted and scanned with HRCT. In a separate experiment, a 10% (w/v deionized water) potassium iodide (KI) solution was injected into a single vessel to determine the utility of this solution as a contrast agent. In addition, fresh internode sections of current year stems were cut from glasshouse-grown plants 24 h before scanning, flushed with deionized water, wrapped in Parafilm, and stored at 7°C for 48 h.

High-resolution computed tomography
Internode sections (5–7 mm) were scanned at the Lawrence Berkeley National Laboratory Advanced Light Source X-ray microtomography facility (Berkeley, CA, USA). The beamline configuration was similar to the standard setup for this technique developed by Kinney & Nichols (1992). The X-rays were emitted from electrons accelerated with a super-bend magnet and pass through a monochromator comprising two multilayer mirrors, which could be altered in angle to select the required X-ray energy from 8 to 45 keV. The stem sample was mounted on an air-bearing stage and X-rays transmitted through the sample interacted with a CdWO₄ single crystal scintillator that fluoresced the shadowgram X-ray image as visible light (1–4, Supporting Information, Fig. S1). This image was then magnified through a series of lenses and relayed onto a 4008 × 2672 pixel charge-coupled device (CCD) camera (#PCO 4000;
The CCD pixel size was 9 μm; thus with a 2x objective, pixel sizes of 4.5 μm of the sample image were mapped onto the CCD sensor. The samples were rotated 180° in the X-ray beam in increments of 0.125°, yielding 1440 different 2D images per sample. A typical scan of a stem section of 5 mm in axial length required c. 20–40 min and generated data at c.19 GB cm⁻³ stem. Samples were scanned in absorption mode, and the reconstructed images were obtained following normalization and the application of a filtered back-projection algorithm.

Raw 2D tomographic projection images were reconstructed using Octopus 8.3 software (Institute for Nuclear Sciences, University of Ghent, Belgium) to create a 3D, 16-bit series or ‘stack’ of tagged image file (.TIF) format files (Dierick et al., 2004). Each TIF image was composed of 3D pixels (volumetric pixel elements, or ‘voxels’), where intensity was based on X-ray attenuation. Each voxel was assigned an x, y and z coordinate in 3D space. All images were processed with Avizo’s ‘Edge-Preserving Smoothing’, an edge-preserving smoothing filter in Avizo 6.2 software (VSG Inc., Burlington, MA, USA), to increase contrast between plant tissue and vessel lumen. Relative mass attenuation coefficients were then calculated using Octopus software to verify the density values present in the HRCT images to distinguish between plant tissue and air- or water-filled vessels.

Manual network analysis

In Avizo, manually selected vessel lumina were segmented away from other tissues using the ‘Label-field’ segmentation editor, and rendered into 3D volume displays using the ‘SurfaceGen’ volume rendering module that integrated the cross-sectional area of each vessel along its length. The spatial data from voxels around the perimeter of a lumen were used to create a triangular 3D mesh displayed as a surface that was freely rotated in virtual space to visualize the vessel network.

In order to characterize intervessel connections, HRCT and scanning electron microscope (SEM) micrographs of identical sections were analyzed visually to confirm the presence of intervessel pits between adjacent vessels. Following HRCT imaging, stem samples were dissected to expose transverse or longitudinal surfaces. The samples were oven-dried at 70°C, sputter-coated with gold, and observed at 5 kV with an FEI/Phillips XL30-SFEG SEM (FEI, Hillsboro, OR, USA). Using the resin-filled vessel for orientation, we measured the actual distances between 363 vessel pairs, with intervessel distances ranging from 3.3 to 140 μm, in SEM images (using Image J software; National Institutes of Health, Bethesda, MD, USA) and noted the presence or absence of intervessel connections (pit fields). These data were used to establish a proximity criterion for the presence of intervessel connections used in both manual and automated network analyses.

In subsequent manual analysis of HRCT images, vessels within dorsal/ventral (D/V) and lateral sectors of a stem cross-section were analyzed separately (Fig. S2). Analyses incorporated all vessels in sectors between adjacent rays, except those most recent (peripheral) ones whose small size made them difficult to resolve as true vessels. Vessel diameters and intervessel connections were determined using the 3D measurement tool in Avizo.

Automation of network analysis

We developed software to automate the analysis of vessel parameters and network connectivity. Although the commercial Avizo software can automatically develop a ‘skeleton’ of connected voxels (Fig. 1a), accurate anatomically based analysis of the HRCT data required additional image processing in Avizo and the development of custom software both to eliminate artifacts arising as a result of scan noise and to resolve intervessel connections. The custom automation was divided into two parts. First, we used components of Avizo’s image analysis modules to create a preliminary network based on voxel intensities in the HRCT images, and secondly, we developed an external program to modify this network, removing noise present in the HRCT images and taking into account known aspects of grapevine anatomy.

In order to identify vessel lumina, image data were first segmented with Avizo’s Arithmetic and LabelVoxel modules, which generated a binary image comprising vessel lumen and nonlumen voxels. The resulting image was compared with the original grayscale HRCT image to determine if any boundary voxels were not captured; these were manually added in order to render the entire vessel boundary accurately. Next, because voxel intensity was not always homogeneous in the lumen, the Fill module in the Image Segmentation Editor was used to ‘fill in’ these areas. Then, Avizo’s DistanceMap, Cast Field, Thinning, TraceLines, and EvalonLines modules were employed to produce a preliminary network of segments (putative vessels) and nodes (putative intervessel connections) (Fig. 1a).

The preliminary network established with the commercial software still contained spurious vessel segments and connections, and the commercial software had no facility to establish criteria for intervessel connections. Therefore, an external program, TANAX (Tomography-derived Automated Network Analysis of Xylem), was developed in the Fortran programming language to: reduce the noise associated with the Avizo-generated network; establish criteria for predicting intervessel connections; and analyze anatomical features of xylem conduits.

In TANAX, first, noise was eliminated from the Avizo-generated network by removing duplicate nodes, duplicate segments, unconnected segments, and segments that started
and ended at the same node. Secondly, all nodes near the (basal) boundary plane of the network domain ($z = z_{\text{min}} = 0$) were located. Thirdly, vessels were reconstructed acropetally on a node-by-node basis. For each step in the reconstruction, all segments attached to a node were evaluated for inclusion/exclusion from the ‘vessel’. Segments were excluded for being dead ends or for being incorrectly oriented in 3D space (e.g. horizontally). Successive segments were selected using the same bulk alignment condition until the vessel terminated or the program reached an end node near $z = z_{\text{max}}$ (Fig. 1b).

Finally, adjacent vessels were analyzed by TANAX for the presence of intervessel connections based on a proximity criterion established in this study. TANAX first determined coordinates for centers of neighboring vessels and then marched from one center toward the other with a step size of the dimension of one voxel, checking to confirm if the current voxel was a boundary voxel. After reaching a boundary voxel at a vessel wall edge for each of the two vessels being examined, the distance between the respective boundary voxels was calculated, and an intervessel connection was established if the distance was less than the proximity criterion stipulated (Fig. 1c i). Owing to the discrete nature of the boundaries, it was observed that this simple algorithm captured many, but not all, connections. Therefore, for intervessel distances less than or equal to twice the value of the criterion, a second algorithm was used to find any intervessel connections that the first algorithm missed (Fig. 1c ii,iii). In the second algorithm, the previously determined boundary voxels from the two neighboring vessels were used to find all of the boundary voxels and measure the distance between each boundary voxel on both vessels (c, bottom), again establishing an intervessel connection if a distance is less than the proximity criterion. Note that for clarity, not all distances measured between boundary voxels on the neighboring vessels are shown (c, bottom). Intervessel connections meeting the proximity criterion are then added to the backbone vessels to create the final network (d). No scale bars are presented because in two-dimensional graphical renderings of three-dimensional structures the scale is not consistent in different dimensions.
voxel on the other boundary was calculated. If the distance between any of the voxels on the first vessel boundary and any of the voxels on the second vessel boundary was less than or equal to the user-defined proximity criterion, an intervessel connection was established (Fig. 1c), and the network assembled (Fig. 1d).

Anatomical calculations were performed on 4.5-mm-long stem internode sections to determine vessel diameters and their radial distribution, change in vessel position in the radial or tangential directions with increasing $z$, and the number and orientation of intervessel connections. The axial vessel path was analyzed by comparing $(x, y)$ coordinates between scans at $z = z_{\text{min}}$ and $z = z_{\text{max}}$. After locating intervessel connections in the $x$–$y$ plane, the orientation of those connections was analyzed by calculating the angles associated with the coordinates of the centers of neighboring vessels. The network connectivity and other anatomical properties of vessels in four sectors were determined using both manual and automated methods for comparison.

**Results**

**Development of HRCT as a diagnostic tool for vessel network analysis**

Based on preliminary tests using 10–35 keV X-ray fluxes, 15 keV was chosen for providing excellent contrast between air, water, resin, and plant tissue. Differences in material density were validated by relative mass-attenuation coefficients calculated from HRCT images using the Octopus software (relative values for resin, plant tissue, and air-filled vessel lumen were 7.9, 4.3 and 0.5, respectively). The $10 \times 5$ mm field of view at the 4.5 $\mu$m resolution allowed the entire cross-sectional area of the grapevine stems to be scanned at a rate of c. 1 cm of stem h$^{-1}$. The high-density silicone resin was easily distinguished from the air-filled lumen of surrounding vessels in the SEM and HRCT micrographs (Fig. 2a,b, respectively). Grayscale HRCT images showed plant tissue as light voxels and air-filled vessel lumen as dark voxels (Fig. 2b).

Intervessel scalariform pits could not be resolved in the HRCT images at the resolution used to capture entire stem section data. To empirically establish when intervessel connections were present, a series of experiments were conducted in which a stem section was imaged by HRCT (Fig. 2b), and then dissected to extract a subsample containing the fiduciary resin marker, which was then observed by SEM (Fig. 2a,c). In this way, we directly observed whether intervessel pits were present for specific vessel pairs (Fig. 2d) in known locations in the HRCT images. An analysis of 363 vessel pairs in which vessels were separated by 14.0 $\mu$m or less (Fig. 3a) showed a clear threshold for the presence or absence of intervessel pits. For vessel lumens separated by 14 $\mu$m or less, 98.3% shared a wall bearing...
intervessel pits. Vessels separated by > 18 μm showed no presence of intervessel pits in their walls (Fig. 3b). In addition, 94.7% of the vessel pairs measured were separated by > 4.5 μm (Fig. 3a), the resolution limit of the HRCT scans. Therefore, vessels with boundaries separated by < 14 μm in HRCT images were classified as interconnected (i.e. sharing intervessel pits).

High-resolution X-ray computed tomography image slices revealed that the proximity of adjacent vessels was not constant along the z-axis. In an example vessel pair evaluated in cross-section at two z-planes, the intervessel distance is greater in plane 1 than in plane 2 (Fig. 4a,b, respectively). Volume rendering of selected vessels up to plane 1 show their contiguous borders (Fig. 4c), and further up to plane 2 their separation (Fig. 4d). Intermittent vessel connectivity was frequently observed. The changing connectivity (displayed in volume renderings as in Fig. 4(e) and Movie S1) could be confirmed by SEM image analysis as described earlier. The HRCT images can be viewed slice-by-slice through any plane, that is, along the y- or z-axis as in Fig. 5(a). The side length of the triangular elements was modified with the Surface Simplifier module to increase or decrease the amount of detail present in the surface of vessel volume renderings, revealing the location of individual vessel elements (Fig. 5a, inset).

Manual and automated vessel network analysis

The data presented here are representative of repeated comparisons analyzing different scans of different stem sections. When manual and automated methods were applied to the same four sectors in a 4.5-mm-long stem internode scan, mean vessel diameters were similar (Table 1). At a sampling frequency of every 10 transverse slices (450 μm apart), both methods found that most vessels had one or more intervessel connections. The inputs to TANAX were then adjusted to employ a greater sampling frequency (every 45 μm), revealing more intervessel connections (76) than the manual analysis (62) or automated analysis with the lower sampling frequency (52) (Table 1). TANAX produces data that are otherwise difficult to measure manually, including: the average angle of drift, the average vessel diameter (with standard deviation); a list of all of the vessels in the analyzed section with vessel diameters and their radial coordinates relative to stem center; the number of vessels connected at any point to each vessel; the total number of intervessel connections (i.e. one connection is whenever a vessel contacts another at any point)

Fig. 3 (a) Distribution of the sampled intervessel distances used for vessel connectivity analysis. (b) The presence or absence of intervessel pit membranes with respect to intervessel distance. Intervessel distance was determined as the minimum distance from one lumen to the adjacent lumen. The light gray shaded area in (b) shows the 4.5 μm resolution limit of high-resolution X-ray computed tomography (HRCT) imaging.

Fig. 4 High-resolution X-ray computed tomography (HRCT) micrographs showing two vessels with changing connectivity through the length of a fresh excised stem sample flushed with air. (a) Transverse view of plane 1 in (e) showing the close proximity of the two neighboring vessels sharing a wall (white arrow, wall thickness = 13 μm). (b) Transverse view of plane 2 in (e) showing increased intervessel distance (arrow, distance between vessels = 25 μm). (c, d) Alternate transverse views of planes 1 and 2 that include vessel volume renderings. (e) Longitudinal view of vessels with an intervessel connection at transverse plane 1 and loss of connection at transverse plane 2. Bars, 150 μm (a–d); 450 μm (e).
The automated method generates these data in a fraction of the time required by the manual method to produce vessel diameters and connections, and the sampling frequency has little impact on the computation time (Table 1). The entire process of performing anatomical analysis, establishing intervessel pit connections, and generating the xylem networks described by the remaining data presented in this study required ~4 h of image preparation time and 2 min of computational time on a 64-bit, quad-core computer with 2.83 GHz processors.

The automated method generates vessel networks from binary images (Fig. 5b left) as a ‘skeleton’, with vertical bars representing the location of the center-point of the vessel lumen and horizontal bars representing areas of intervessel connectivity (Fig. 5b right). These ‘networks’ can be overlaid with a transparency of the volume renderings to display the vessel lumen and the network skeleton simultaneously (Fig. 5c) and to allow the user to visualize intervessel connections in the context of vessel dimensions (see also Movie S1).

**Table 1** Comparison of manual vs automated vessel network analysis of high-resolution X-ray computed tomography (HRCT) images from dorsal/ventral vessels in a 4.5-mm-long internode section of grapevine (*Vitis vinifera*).

<table>
<thead>
<tr>
<th>Analysis method</th>
<th>Manual</th>
<th>Automated</th>
<th>Automated</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>SF (µm)</td>
<td>450</td>
<td>450</td>
<td>45</td>
</tr>
<tr>
<td>(C_v)</td>
<td>0.86 ± 0.77</td>
<td>0.63 ± 0.74</td>
<td>1.15 ± 0.89</td>
</tr>
<tr>
<td>Connections = 0 (%)</td>
<td>38</td>
<td>48</td>
<td>24</td>
</tr>
<tr>
<td>Connections = 1 (%)</td>
<td>39</td>
<td>37</td>
<td>54</td>
</tr>
<tr>
<td>Connections &gt; 1 (%)</td>
<td>23</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>(D_v) (µm)</td>
<td>67.1 ± 18.2</td>
<td>65.0 ± 26.7</td>
<td>65.0 ± 26.7</td>
</tr>
<tr>
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<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Analysis time (h)</td>
<td>16</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Values are means ± SD unless otherwise noted.

\(n\), number of sampled vessels; SF, sampling frequency, axial distance between sampling points; \(C_v\), mean number of connections per vessel; \(D_v\), mean vessel diameter. Image preparation time includes data transfer, cropping and image processing. Analysis time, automated or manual computation time required to analyze the selected vessels.

Spatial distribution of vessels and their connections from TANAX analysis

From automated analysis of representative D/V and lateral vessel sectors (Fig. S2), vessel diameters ranged from 18 to 138 µm in D/V zones and from 17 to 77 µm in lateral zones (Fig. 6a). The automated method discriminated the smaller mean diameters and axial ‘drift’ (AD) of vessels in lateral zones compared with those in D/V zones (Table S1). Mean vessel diameter in D/V zones (65.0 µm) was significantly greater (\(P < 0.001\)) than in lateral zones (37.3 µm). The axial drift was 2.8° and 2.0° for D/V and lateral vessels, respectively. Lateral vessels were displaced away from the stem center compared with D/V vessels.

**Fig. 5** High-resolution X-ray computed tomography (HRCT) micrographs showing how three-dimensional (3D) voxels were used to display virtual slices through plant tissue, produce volume renderings, or create anatomical maps. (a) Longitudinal and transverse grayscale HRCT slices show light voxels as stem tissue, while dark voxels represent the air-filled vessel lumen. Each axis is labeled in the \(x\)-, \(y\)- or \(z\)-plane. Vessel volume renderings are composed of a 3D ‘mesh’ of triangular surfaces (inset), revealing the 3D characteristics of individual vessels. (b) Grayscale images were then converted to binary images to prepare the dataset for network analysis. Results of the network analysis are presented as a skeleton of line segments (yellow) at the vessel lumen center and nodes (gray circles and yellow horizontal lines) at pit connection locations (inset). (c) Vessel volume renderings and the network skeleton were displayed simultaneously to compare both methods for analyzing intervessel connectivity. Bar, 150 µm.
A pattern of vessel diameter distribution was not apparent in lateral vessels, but it was clear that the largest $D/V$ vessels occurred midway in the xylem and thus were not produced first (closest to the stem center) (Fig. 6a). As rays diverged away from the center of the stem, more cross-sectional area in each sector was occupied by $D/V$ vessels, a trend visible in the distribution of vessel position from the stem center (Fig. 6b). There was no apparent correlation between the number of vessels within a sector and the vessel diameter or vessel drift in either zone.

Vessels in both zones showed a maximum in intervessel connections at an intermediate position, and less frequent connections near the center and epidermis (Fig. 6c), but no clear dependence upon vessel diameter (Fig. 7). Intervessel connections were primarily aligned in the radial (0°, oriented to the shortest distance from the stem center to the epidermis) and tangential directions (± 90°), with the highest frequency of connections at −10° to 0° for $D/V$ vessels (Fig. 8a), and between −20° and +20° for lateral vessels (Fig. 8b). In both zones there was an increase in connections at ± 80° as a result of connections between parallel vessel sectors. Vessels within lateral sectors were slightly offset from each other, leading to the wider range of connection angles. Shared pit area between vessels was highly variable. As a result, the standard deviation often exceeded the mean in both $D/V$ and lateral vessel sectors (Table S2). The smaller lateral vessels were more highly interconnected than the larger $D/V$ vessels (cf. Fig. 8a,b).

Imaging hydrated, dehydrated, and live plant tissue

In fresh stem tissue, HRCT was used to differentiate between vessels filled with air or water. When air was
pushed through the stem sections at low pressure, most vessels were visibly empty (Fig. 9a,b), indicating that the majority of the vessels in this section did not contain an end wall. After being flushed with water, the vessels were clearly filled and vessel wall edges remained visible (Fig. 9c,d). In both hydrated and dehydrated stems, vessel parenchyma, fibers, cambium, and phloem cells were not easily identified. The pith was conspicuous in fresh and live plant tissue, and many of the cells occupying the pith were filled with air or water vapor (Fig. 9).

**Discussion**

When properly calibrated, HRCT provides unprecedented access to xylem conduits at a spatial and temporal resolution that facilitates the study of vascular organization in plants. The resulting images and volume renderings can be thought of as a 3D, nondestructive SEM, albeit at a lower resolution. Selected vessels or the entire network are easily visualized by freely rotating the volume renderings in 3D space, or viewing serial slices in any plane or orientation (Movie S1). The integration of visualization tools (HRCT and SEM) with custom software allows users to quantify vessel network components and their connectivity. We expect this to be a first step in an iterative process of building accurate physical and mathematical models to explore the physiological implications of the spatial organization of the xylem. However, further developments will be required to extend this work to analysis of an entire shoot.

The automated analysis identifies vessels and generates vessel diameters, and the orientations of vessels and their connections at user-defined sampling rates along the z-axis of stacked HRCT grayscale images of grapevine stem internodes. At a sampling rate 10 times greater than a manual analysis, the time required to use TANAX was c. 1% of that necessary for manual analysis. As more scans are incorporated, for example, for additional internodes and nodes, this
advantage will multiply. Furthermore, our experience is that the data generated by the automated method are more reliable than the commercial software volume rendering or our manual analysis, in part because the latter involves user-subjective components in determining vessel lumen and connections, and depends on visual assessment of voxel intensity rather than the intensity data contained in the original TIF files.

It was difficult to resolve intervessel pits and vessel endings in our HRCT images. However, we found that in grapevine, intervessel pits were present whenever the boundaries of adjacent vessels were within 14 μm. This allowed us to create a proximity criterion for mapping intervessel connections from HRCT images. Although we did not address vessel endings in the short samples in this study of long-veinsed grapevine, higher-resolution CT of longer sections may reveal vessel ending distributions (Mayo et al., 2010). Van den Bulcke et al. (2008) analyzed conifer wood samples at a 0.79 μm resolution and visualized the presence of a fungal pathogen, tracheid endings, and intertracheid pits. Current-generation submicron resolution scans are limited by a small field of view (500 × 250 × 400 μm), thereby reducing the utility of those scans for the analysis of whole vessel networks, even when multiple scans are merged together. Some increase in signal:noise ratio of HRCT scans over what is shown here may be realized by decreasing the angular increment (i.e. increasing the number of projection images), averaging multiple projection exposures, and optimizing both sample preparation and X-ray energy. Laboratory-based CT systems are available; however, the synchrotron HRCT facilities provide a greater choice of wavelengths and collimated light that increases signal:noise ratio and allows optimization of image contrast (Paulus et al., 1999). Synchrotron HRCT systems are also faster at imaging because the flux density is much higher than laboratory-based systems, thereby reducing scanning time for a 5 mm stem from 4 h or more on a laboratory-based system to 20 min.

The analysis of xylem conduits in grapevine stem confirmed the suggestion by Stevenson et al. (2004) that vessel diameter is greater in D/V zones than in lateral zones that give rise to lateral shoots, leaves, tendrils, and fruit. It was also clear that the distribution of vessel diameters did not follow a typical pattern of large diameters early followed by smaller diameters later, towards the epidermis. The largest vessels appeared in an intermediate position in the D/V zones. Smaller diameter vessels in the lateral zones were more closely spaced and interconnected than large-diameter vessels in the D/V zones.

TANAX provided considerable information about the intervessel connections. In these young, current-season V. vinifera stems, most intervessel connections were oriented radially (i.e. outward from the stem center) rather than in a tangential plane between vessels. V. vinifera rays are multiseriate (Sun et al., 2006), and this limits the potential for tangential movement of vessels and, hence, water, solutes, and even xylem-dwelling pathogens in young shoots. Vessels were observed passing through rays establishing trans-ray connections, although this was rare. Divergence of rays with increasing wood development in succeeding years may allow for increasing cross-sectional area to be occupied by vessels, and correspondingly more tangential communication. Grapevine is well known for its long, wide vessels (Zimmermann & Jeje, 1981), with the effect that embolism can significantly influence water transport (Choat et al., 2010). By isolating the vessels both within a sector and between rays, the stem becomes modular, with vessels or sectors substantially hydraulically isolated from its neighbors. The differences in connectivity between D/V and lateral vessels also suggest that there is some selective advantage to integrating vessels leading to leaves, fruit, and tendrils, and isolating vessels that pass through nodes.

Carlquist (1984) included the Vitaceae in a group of dicotyledonous plant families possessing vessels that are nearly always found in groups of two or more. Tyree & Zimmermann (2002) stated that vessels that are together ‘invariably’ share intervessel pitting. Our data show that such grouping and the meaning of ‘together’ can now be functional and quantitative (at least in V. vinifera) based on vessel-to-vessel proximity, and some vessel connections are intermittent. Intermittent vessel contact does not appear to be limited to V. vinifera, and was documented in Fraxinus excelsior by Burggraaf (1972). Thus, vessels may appear to be together in cross-section but are not connected. Although some vessels did not have connections in the short stem sections studied here, this is not interpreted to indicate a high frequency of isolated vessels. Also, in cross-section, vessel dimensions and connections were not consistent among sectors (between rays). Therefore, it is important to determine the minimum amount of tissue that must be analyzed to form an accurate representation of the stem as a whole (Kitin et al., 2004). The probability of systemic embolism spread appears to increase with increasing intervessel contact (Tyree & Sperry, 1989; Wheeler et al., 2005). Our data show that the standard deviation of intervessel contact area was greater than the mean, indicating that the contact areas must be skewed to higher values and that the mean would not be representative of any particular place in the network. We now have the ability to locate and quantify intervessel connections in the stem and from that develop accurate models to predict how the distribution of connections influences the spread of embolisms.

Grapevine may prove to be a good system for developing CT methods in the study of xylem organization and function. Many vessels are wide, and, as shown here, there is a clear proximity threshold for the presence or absence of intervessel connections. Contrary to the convoluted xylem organization of ash species (Fraxinus excelsior and F.
lanuginose) observed by Burggraaf (1972) and Kitin et al. (2004), respectively, the vessels of grapevine remained approximately parallel through the length of the stem section. The lack of axial drift in these vessels is an advantage for automated network analysis. Grapevine conduits are highly connected and physiologically interactive with adjacent cells in producing tyloses (Sun et al., 2007) and gels (Sun et al., 2008) and in refilling embolisms (Brodersen et al., 2010). In addition, it is already established that environmental perturbations such as shade (Schultz & Matthews, 1993) and water deficits (Lovisolo & Schubert, 1993) alter some aspects of xylem vessel development. HRCT can be applied to resolve the full impact of those environments on the xylem network structure, and, ultimately, its function.

Furthermore, fresh tissue (present study) or intact grapevine plants can also be scanned to reveal the presence or absence of vapor embolisms. This noninvasive technology was recently used to show the refilling process in individual embolized vessels of grapevine (Brodersen et al., 2010). No evidence of tissue damage was visible in the stems used here, nor has any evidence developed in the plants used previously (Brodersen et al., 2010), although this has not been the case in all species tested (Kim & Lee, 2010). Thus, several further applications of HRCT are envisioned, including quantifying whether the pattern of embolisms among vessels is in accordance with the network interconnections, and quantifying the degree of embolism and of refilling per se associated with hydraulic assays for percent loss of conductivity (PLC) curves. Schultz & Matthews (1993) suggested that in droughted grapevine, xylem pressure might go lower and embolism spread more in petioles than in stems, leading to loss of leaves instead of loss of shoots. Using different but in both cases invasive techniques, Choat et al. (2005) and Hukin et al. (2005) reported evidence that this is the case in other dicot tree species. However, others, (e.g. McElrone et al., 2004) maintain that roots are more susceptible to embolism than stems. In grapevine, these ideas could be tested by noninvasive HRCT scanning of the various organs of the same plants during water deficits.

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References


Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Schematic illustration of the X-ray path.

**Fig. S2** Transverse high-resolution X-ray computed tomography (HRCT) image of a *Vitis vinifera* internode showing the dorsal (D) and ventral (V) sectors of vessels that pass through nodes, and the lateral (L) vessel sectors that lead to leaves and tendrils.

**Table S1** Automated analysis of vessel parameters from dorsal/ventral vs lateral files

**Table S2** Comparison of total intervessel pit area shared between lateral vessels

**Movie S1** Movie of three-dimensional volume renderings of xylem vessels and virtual serial sections though a grapevine (*Vitis vinifera*) stem tissue.

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