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Research

Rapid report

High-resolution computed tomography reveals dynamics of desiccation and rehydration in fern petioles of a desiccation-tolerant fern

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Summary

- Desiccation-tolerant (DT) plants can dry past −100 MPa and subsequently recover function upon rehydration. Vascular DT plants face the unique challenges of desiccating and rehydrating complex tissues without causing structural damage. However, these dynamics have not been studied in intact DT plants.
- We used high resolution micro-computed tomography (microCT), light microscopy, and fluorescence microscopy to characterize the dynamics of tissue desiccation and rehydration in petioles (stipes) of intact DT ferns.
- During desiccation, xylem conduits in stipes embolized before cellular dehydration of living tissues within the vascular cylinder. During resurrection, the chlorenchyma and phloem within the stipe vascular cylinder rehydrated before xylem refilling. We identified unique stipe traits that may facilitate desiccation and resurrection of the vascular system, including xylem conduits containing pectin (which may confer flexibility and wettability); chloroplasts within the vascular cylinder; and an endodermal layer impregnated with hydrophobic substances that impede apoplastic leakage while facilitating the upward flow of water within the vascular cylinder.
- Resurrection ferns are a novel system for studying extreme dehydration recovery and embolism repair in the petioles of intact plants. The unique anatomical traits identified here may contribute to the spatial and temporal dynamics of water movement observed during desiccation and resurrection.

Introduction

Desiccation-tolerant (DT) plants can survive near-complete water loss and subsequently recover following rehydration (Alpert, 2005). Desiccation tolerance allowed early plants to colonize dry land before the evolution of a cuticle, stomata, and a vascular system (Oliver *et al.*, 2000). While today most plants remain DT at the seed or spore stage, relatively few vascular plants have re-evolved DT in their vasculature-dependent leaves, stems, and roots (Oliver *et al.*, 2000). Such plants are aptly named 'resurrection plants' for this ability to recover from apparent death. Resurrection plants persist in dry habitats, including rocky outcrops with shallow soil (Porembski & Barthlott, 2000; Holmlund *et al.*, 2016). Desiccation tolerance may allow plants to survive in regions affected by seasonal drought, such as the mediterranean-type chaparral ecosystem (Holmlund *et al.*, 2016) and other arid or semi-arid ecosystems (Gaff, 1977, 1987; Gaff & Latz, 1978). Desiccation tolerance of the leaves, stems, and roots (vegetative DT) has evolved several times, allowing diversity and distinction among groups of DT plants (Gaff & Oliver, 2013). For instance, *Pleopeltis polypodioides* is a DT fern that thrives as an epiphyte in subtropical moist regions, albeit in a dry epiphytic habitat.

In addition to orchestrating revival processes at the molecular and cellular levels, vascular DT plants must also transport water to dry distal tissues with varied composition and function. Thus, vascular DT plants face three challenges unique to vascular plants. First, they need to experience orderly desiccation of distinct tissue types (e.g. xylem, phloem, chlorenchyma, parenchyma) in order to

prevent damage during desiccation. A combination of tough and flexible tissues may facilitate a successful transition to the desiccated state. Second, vascular DT plants also need to resurrect distinct tissue types in a specific and orderly manner. Finally, they need to restore hydraulic flow through xylem conduits in order to transport water to distal desiccated tissues lacking abundant access to water.

Studies of the mechanisms of DT whole-plant recovery have been limited either by the need to cut the plant open to observe anatomical changes or by the need to infer internal processes from external physiological measurements (Sherwin *et al.*, 1998; Schneider *et al.*, 2000). Such invasive and destructive techniques have the potential to introduce artifacts. While these destructive studies have led to important insights into DT, a complement to such studies is to use advanced imaging techniques such as high-resolution micro-computed tomography (microCT) that uses tissue penetrating X-rays to visualize the internal details of a plant without cutting it open and disturbing function. In the last decade, microCT technology has developed and has been used to image xylem structure, as well as the reversal of gas embolism inside xylem conduits of woody plants (Brodersen *et al.*, 2010, 2012). Here we report that microCT is also a useful tool to visualize the dynamics of desiccation and resurrection in the stipes of DT ferns.

Resurrection in vascular DT ferns represents an extreme case of embolism repair, since the xylem conduits are completely gas-filled in a deeply-desiccated state (< −100 MPa). In this study, we used microCT to characterize the desiccation and resurrection processes in the stipes of intact resurrection ferns. Given the seasonal water potentials reported in Holmlund *et al.* (2016), we hypothesized that water under tension in xylem conduits would cavitate early in desiccation (*c.* −1 to −3 MPa), while solutes in living phloem and parenchyma cells would retain some water when desiccated. We also hypothesized that xylem conduits would rehydrate before phloem cells, because gas-filled xylem conduits may provide less resistance to water flow than desiccated living cells containing membranes and organelles. Therefore, we predicted that the xylem tissue would be the first to desiccate and the first to rehydrate.

Additionally, vascular DT ferns likely have differences in chemical composition among the distinct tissue types found in stipes to regulate desiccation and resurrection. Because of their pivotal location for water transport between soil and leaves, stipe tissues were examined for any unique traits that might explain the desiccation and rehydration patterns found with microCT. We used staining combined with light and fluorescence microscopy to identify the chemical composition of tissues inside the stipe. Of particular interest were cellular compounds that confer toughness (lignin) or flexibility (pectin), as well as cellular structures that impede the flow of water, such as the Casparian-like strip in the endodermis.

Materials and Methods

Plant material

We grew individuals of *Pentagramma triangularis* (Kaulf.) in a glasshouse at the University of California, Santa Cruz, CA, USA, from locally obtained spores. Plants were grown in 7.5 cm pots under partial shade with regular watering. Plants dried naturally during the desiccation experiment, and each of the 15 plants was only imaged one time with microCT. A different group of plants was used for the resurrection experiment. These plants dried naturally before the experiment with no prior exposure to X-ray radiation. During the resurrection experiment, we watered the plants at the roots and sealed them inside a plastic bag to increase the humidity. To reduce variation in recovery dynamics, we consistently resurrected plants under low light (photosynthetic photon flux density (PPFD) ≈ 15 μmol m^{-2} s^{-1}). During resurrection, we imaged each of 10 plants one to four times. Images were timed to capture the full range of hydration, starting with desiccated plants (pre-watering) through rehydrated plants (up to 25 h post-watering). The ferns resurrected similarly regardless of how many times they were imaged, and no signs of radiation damage were observed (Petruzzellis *et al.*, 2018). In several plants, we conducted repeat scans to confirm expansion of the outer stele early in the resurrection process, since there was variation in the size of the dry stele (Supporting Information Fig. S1).

High-resolution micro-computed tomography (microCT)

During desiccation and resurrection, we imaged the stipes of intact plants using high-resolution microCT (also abbreviated as HRCT; Bruker Corporation, Skyscan 2211, Kontich, Belgium). To avoid root damage, the entire contents of each pot were wrapped in parafilm and attached to a rotating pedestal inside the scanner. We stabilized fronds by securing them with plastic wrap to a bamboo stick, allowing one to four stipes to be imaged on each plant. Stipes were scanned 7 cm above the root base at 1–3 μm resolution, with most scans lasting 10–20 min. We took longitudinal images every 0.15°–0.25° over a range of 180°, and the X-ray energy used was 40 kV and 600 μA. From these images, we built three-dimensional (3D) reconstructions of each stipe using InstaRecon software (InstaRecon, Champaign, IL, USA). Distinct tissue types became apparent based on differences in X-ray absorption, including the cortex, phloem/chlorenchyma layer, and gas-filled xylem (Fig. 1). A two-dimensional (2D) cross-section was extracted from the middle of each 3D reconstruction to measure relative area of each tissue type (CTAn software, Bruker Corporation, Billerica, MA, USA).

After the stipes appeared fully resurrected as seen using microCT, we wished to confirm that the water in the xylem conduits was moving (i.e. the conduits were functional, not just water-filled). We excised two resurrected stipes under water and placed them into 150 mM iohexol solution for 1 to 2 h while illuminating them with light (PPFD ≈ 200 μmol m^{-2} s^{-1}) (Pratt & Jacobsen, 2018). Iohexol is an X-ray dense molecule with a topological polar surface area of 2 nm^2, small enough to pass in solution through xylem pit membranes but large enough to stay in the apoplast (Pratt & Jacobsen, 2018). Thus, transpirational uptake of iohexol solution confirmed that most (and possibly all) of the refilled xylem conduits were capable of transporting water (Fig. 1h).

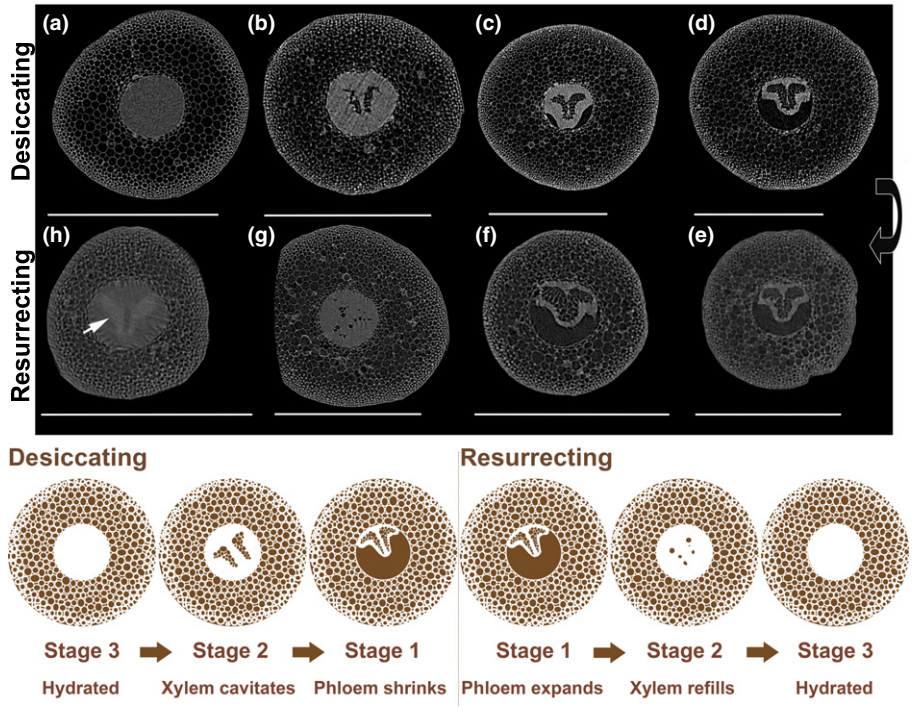

Fig. 1 Representative micro-computed tomography (microCT) transverse images of *Pentagramma triangularis* stipes during desiccation and resurrection (a–h). Bars, 1 mm. Before desiccation, mature, hydrated stipes showed desiccated cortex tissue surrounding a fully hydrated vascular cylinder (a). Early in desiccation, the xylem conduits became gas-filled (b). Subsequently, the living tissues in the vascular cylinder (phloem and chlorenchyma) compressed (c). Fully desiccated stipes showed gas-filled xylem and a shrunken vascular cylinder, leaving a gap between the vascular cylinder and cortex (d, e). Early in resurrection, the phloem and chlorenchyma expanded (f). Later, xylem conduits refilled (g). Fully resurrected stipes resembled mature, never-desiccated stipes (h). Resurrected xylem conduits functioned in water transport, since iohexol solution can be seen in refilled xylem conduits (white region, indicated by arrow in h). A cartoon illustrating the stages of desiccation and rehydration is provided. Stage 3 represents fully hydrated stipes engaged in water transport, as in (a) and (h). Stage 2 includes stipes experiencing xylem cavitation or partial refilling, as in (b) and (g). Stage 1 includes the shrinking or expanding of living tissue inside the vascular bundle (phloem and parenchyma), as in (c–f).

Leaf water potential

Throughout desiccation and resurrection, we measured leaf water potential (Ψ_{leaf}) of individual plants at the time of microCT imaging. Thus, Ψ_{leaf} was measured twice in most plants during the resurrection experiment: once in the desiccated state and once after the last image was taken of that plant resurrecting. (No stipes were imaged after the leaf was removed.) We measured Ψ_{leaf} using WP4C dew point hygrometers that are capable of measuring samples from zero to −300 MPa (Decagon Devices, Pullman, WA, USA). We excised leaves from stipes shortly after the plant was imaged with microCT or from an adjacent frond immediately before imaging. We sealed the leaf tissue in two plastic bags for any interim time. The leaf tissue was quickly cut into narrow strips (< 1 mm) inside a humid bag and then placed inside the chamber of the dew point hygrometer for the reading. The hygrometer internally sealed the chamber adjacent to a small mirror and adjusted the temperature of the mirror until the fogging point (dew point) was determined. Equilibration time was usually 15–30 min. Cutting the leaf tissue into narrow strips expedited equilibration time.

Furthermore, since the desiccation time for each plant was variable, Ψ_{leaf} provided a better predictor of changes in tissue water status than time since watering (Fig. 2c–j). Unless distinct differences were apparent among fronds on a single plant (e.g. drastic difference in height or resurrection status), Ψ_{leaf} of a single leaf was measured to represent all stipes on the plant at that time.

Light and fluorescence microscopy

We examined stipe cross-sections using light and fluorescence microscopy to identify traits that may facilitate the desiccation and resurrection processes (Fig. 3). Fresh cross-sections were prepared by hand using razor blades (GEM single edge stainless steel PTFE-coated blades; Electron Microscopy Sciences, Hatfield, PA, USA) to cut thin sections that were then mounted in water for microscopic examination. Other fern stipes were placed in 10% neutral buffered formalin solution (Fisher Scientific, Protocol, Kalamazoo, MI, USA), for a minimum of 48 h before being sectioned. Then we viewed samples either unstained or following staining with phloroglucinol–hydrochloric acid (HCl) or acid fuchsin with stains prepared following Ruzin (1999). Lignin was identified by staining with phloroglucinol–HCl (Jensen, 1962; Fig. 3e,f), and glycoproteins were stained with acid fuchsin (Fulcher & Wong, 1982; Crews *et al.*, 1998; Fig. 4g,h). Cross-sections were viewed either using light microscopy or fluorescence microscopy (Zeiss Stereo Discover V.12 with Axiocam HRc digital camera; Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). For

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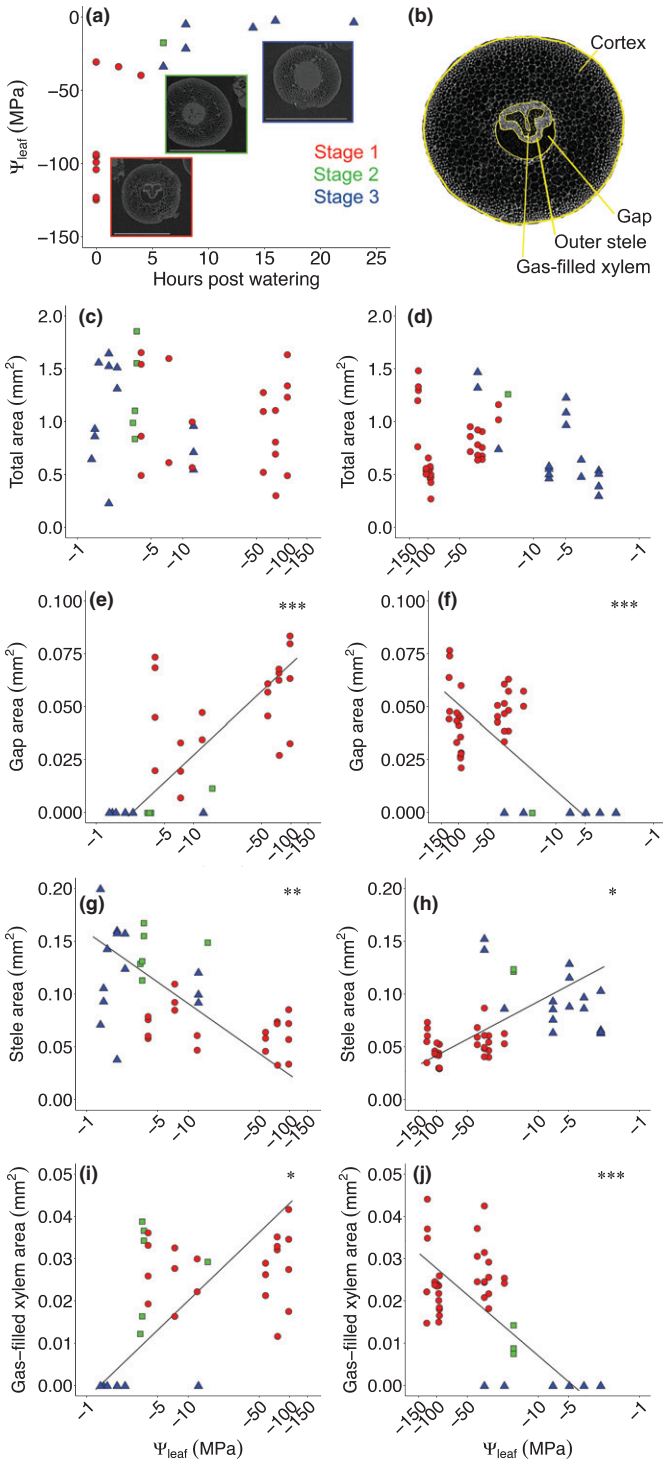

Fig. 2 Changes in leaf water potential (Ψ_{leaf}) with time since watering during the resurrection process (a). Point color and shape correspond to the stage of resurrection, based on observations in Fig. 1. Stage 1 (red circles) includes plants with desiccated stipes and stipes starting to resurrect (expanding phloem and chlorenchyma). Stage 2 (green squares) includes plants with stipes showing partly refilled xylem. Stage 3 (blue triangles) includes plants with fully resurrected stipes. Tissue types distinguishable using micro-computed tomography (microCT) are identified in (b). Changes in transverse stipe tissue area with Ψ_{leaf} were observed during desiccation (c, e, g, i) and resurrection (d, f, h, j). No changes were observed in total transverse area with log Ψ_{leaf} (c, d), but area of the gap, stele (vascular cylinder), and gas-filled xylem correlated with log Ψ_{leaf} (e–j; standardized major axis regression; significant when $P < 0.05$). Asterisks indicate significance level (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The standardized major axis regressions were calculated using the mean values of all stipes in a plant (one to four stipes per plant, $n = 15$ means for desiccation experiment, $n = 16$ means for resurrection experiment). However, data points for individual stipe values are plotted to illustrate natural variation among stipes (c–j).

each sample preparation (fresh vs fixed) or stain (no stain, phloroglucinol–HCl, or acid fuchsin), we examined at least six and often many more sections from different stipes.

Statistical analyses

We conducted all statistical analyses using R (v.3.5.0; R Core Team, 2018). Ψ_{leaf} was log-transformed to fit assumptions of normality. When testing for correlation, we fit data with a standardized major axis regression (SMATR package; Warton *et al.*, 2012). Although we calculated the regression using the average values from all stipes on a plant (one to four stipes per plant), we plotted the individual stipes to show variation (Fig. 2c–j). Regressions were considered significant at $P < 0.05$.

Results

Vascular anatomy

MicroCT revealed four important anatomical regions in the stipes of *P. triangularis*: epidermis; cortex; stele, including the vascular cylinder and gas-filled xylem conduits in desiccated samples; and a gap between the cortex and stele in desiccated samples (Figs 1, 2b). In the transverse section, cortex tissue comprised the majority of the stipe, including the outer sclerenchyma layer and interior parenchyma. This cortex tissue desiccated during development and never rehydrated during resurrection. The vascular cylinder (stele) included chlorenchyma, phloem, and xylem. The phloem and chlorenchyma tissue were indistinguishable using microCT, but these tissues could be identified using light and fluorescence microscopy (Fig. 3). Fluorescence microscopy revealed a Casparian-like strip within the endodermal layer surrounding the vascular cylinder, but this layer could not be distinguished from the other tissues using microCT (see Fig. 3).

The stages of development affected which tissues were gas-filled. The stipes of mature fronds had dry cortex tissue, whereas young stipes had hydrated cortex tissue (image not shown). Hydrated cortex cells would have been necessary for early stipe growth because turgor pressure is a prerequisite for cell enlargement. Interestingly, little to no native gas embolism in xylem conduits was observed in any mature fronds before the start of desiccation (Fig. 1a).

Desiccation

We desiccated individuals of *P. triangularis* in pots to observe the tissue responses in their stipes. Based on all the images collected during the desiccation process, we identified three clearly differentiated stages of desiccation (summarized in Fig. 1). First,

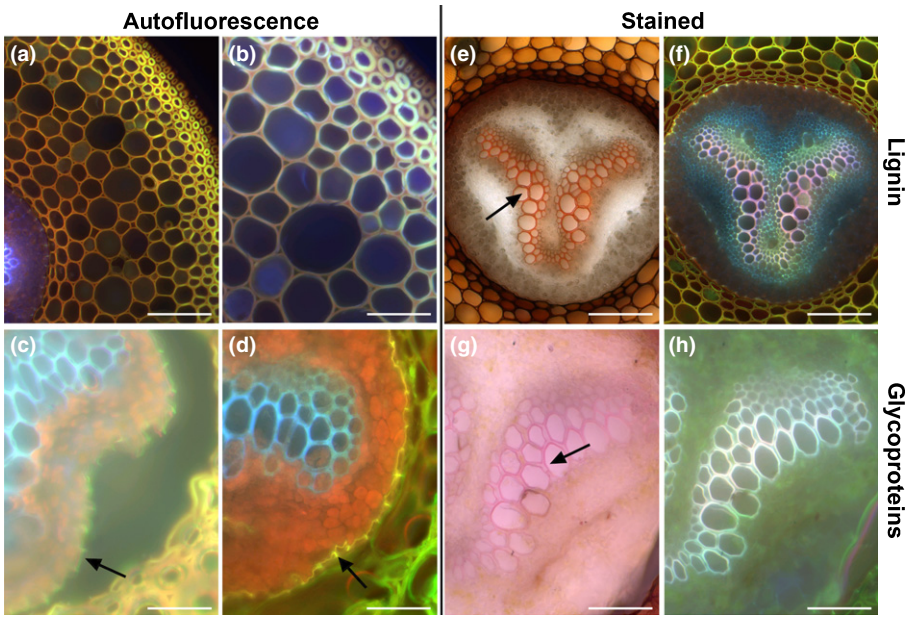

Fig. 3 Transverse sections of fresh (a–d) and fixed (e–h) *Pentagramma triangularis* stipes. Bars: (a, e, f) 100 μm; (b, c, d, g, h) 50 μm. Autofluorescence revealed that the cortex tissue is slightly lignified (blue) and likely contains cellulose (yellow) (a, b). The appearance of the vascular cylinder changed slightly from the desiccated state (c) to the resurrected state (d), but both images showed densely packed chloroplasts in the parenchyma surrounding the xylem and an endodermal layer (arrows) surrounding and attached to the vascular cylinder. The xylem conduits likely contain both lignin (blue) and cellulose or pectin (yellow). Staining with phloroglucinol–HCl revealed some lignin in the xylem conduits and cortex cells, but less lignin than is typical in woody angiosperms, shown with brightfield (e) and fluorescence (f). Staining with acid fuchsin revealed glycoproteins lining the interior of the xylem conduits, shown with brightfield (g) and fluorescence (h).

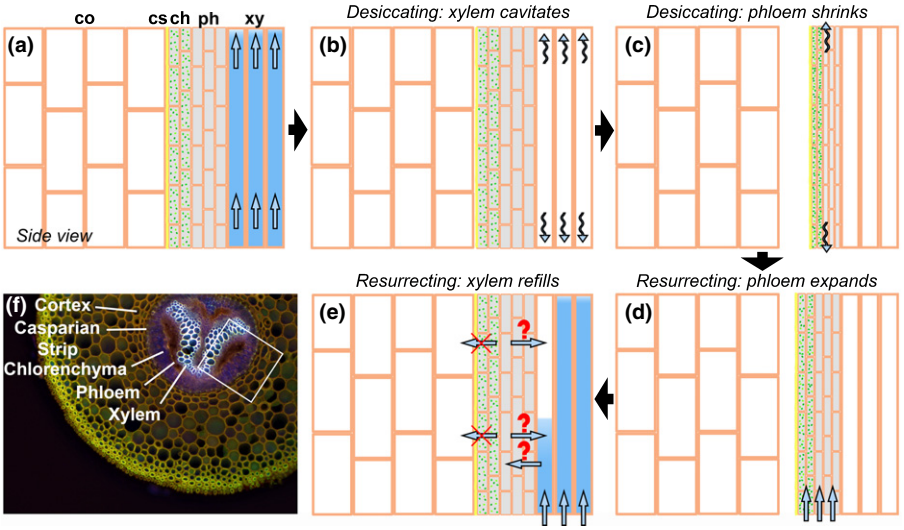

Fig. 4 Conceptual diagram summarizing the findings in this study. Fully hydrated *Pentagramma triangularis* plants show desiccated cortex tissue (co) surrounding the Casparian-like strip (cs) and the hydrated chlorenchyma (ch), phloem (ph), and xylem (xy) tissue (a). During the early stages of desiccation, the stipe xylem conduits become gas-filled, reducing water flow to the leaves and also to the stipe phloem and chlorenchyma tissue (b). Later in desiccation, the stipe phloem and chlorenchyma tissue shrinks as it loses water (c). During the early stages of rehydration, the stipe phloem and chlorenchyma tissue expands as it begins to rehydrate (d). Later in rehydration, the stipe phloem and chlorenchyma tissue becomes fully expanded and the xylem conduits refill (e). Further studies are needed to fully resolve the dynamics between these three tissues during refilling. Since the phloem and chlorenchyma rehydrate first, it is possible that these tissues aid in xylem refilling (arrows in e). However, it is also possible that xylem refilling by capillary action or root pressure aids in the complete rehydration of the phloem and chlorenchyma tissue (arrows in e). A fixed cross-section of a representative stipe is shown with all tissue types labeled (f). co, cortex tissue; cs, Casparian-like strip; ch, chlorenchyma; ph, phloem; xy, xylem.

consistent with our initial hypothesis on desiccation sequence, embolized xylem conduits became clearly visible early in the desiccation process (Fig. 1b). Second, subsequent to initial embolism of xylem conduits, phloem and chlorenchyma tissues shrunk, progressively pulling the outermost endodermal layer of the stele away from the cortex, thus forming a widening gap

between the collapsing vascular cylinder and the cortex (Fig. 1c). We observed large quantities of embolized conduits in all images with shrinking phloem and chlorenchyma tissue (Fig. 1c), indicating that the majority of cavitation in the xylem likely occurs before the living tissue condenses. Third, additional desiccation caused further compression of the phloem and chlorenchyma (Fig. 1d).

Resurrection

We watered the soil of intact, desiccated individual plants in pots and kept them inside sealed bags to maintain a humid environment. Desiccated fronds showed gas-filled cortex and xylem cells and shrunken phloem and chlorenchyma (Fig. 1e). However, in contrast to expectation, the phloem and chlorenchyma rehydrated first, not the xylem, filling the gap between the vascular cylinder and cortex (Fig. 1f). Early expansion of the phloem and chlorenchyma was most apparent in several instances where fronds were re-imaged early in resurrection (Fig. S1). Next, xylem conduits refilled, evident in images that show a mix of both water-filled and gas-filled conduits (Fig. 1g). Lastly, fully resurrected stipes closely resembled never-desiccated stipes in appearance (Fig. 1a,h).

To determine whether resurrected xylem conduits were functional (moving water), we excised resurrected fronds under water and fed iohexol solution to the cut end of the stipe for 1–2 h while plants were transpiring (Pratt & Jacobsen, 2018). The imaged stipes confirmed that water (iohexol solution) was moving through the refilled xylem conduits (Fig. 1h). The cortex tissue showed no signs of rehydration (Fig. 1f–h).

Changes in tissue area with leaf water potential

The Ψ_{leaf} ranged from −125 MPa (desiccated) to *c.* −1 MPa (hydrated). Furthermore, Ψ_{leaf} of resurrecting fronds increased with time since watering ($P < 0.0001$, Fig. 2a). However, Ψ_{leaf} of desiccating fronds was highly variable, possibly because the leaf tissue of this species is hydraulically disconnected from the stipe following complete xylem embolism *c.* −3 MPa (H. Holmlund, unpublished). The Ψ_{leaf} of hydrated plants was consistent with the normal range of Ψ_{leaf} in the field (*c.* −1 to −2.5 MPa; Holmlund *et al.*, 2016). The transverse area of each region within the stipe (total area, gap between cortex and vascular cylinder, stele, gas-filled xylem; Fig. 2b) was measured at varied stages in the desiccation and resurrection processes. The total transverse area of the stipe did not change with decreasing or increasing Ψ_{leaf}, consistent with our observation that the cortex desiccates during development and does not refill during resurrection (Fig. 2c,d). By contrast, other transverse regions of the stipe (gap, stele, and gas-filled xylem) changed area with decreasing or increasing water potential (Fig. 2e–j).

Tissue chemical composition

We used light and fluorescence microscopy to elucidate the role of each tissue in producing the patterns observed with microCT. We observed stipe cross-sections using light microscopy, reactivity to different histological stains (phloroglucinol–HCl for lignin, acid fuchsin for glycoproteins), autofluorescence, and a combination of stains and fluorescence (Fig. 3). Autofluorescence revealed that the cell walls within the cortex tissue included several constituents (Fig. 3a,b). Lignin was present in the cell walls of cortex parenchyma surrounding the stele, as well as in the cell walls of the sclerenchyma tissue near the epidermis (yellow walls, Fig. 3a,b; red stain, Fig. 3e). Fluorescence also indicated the presence of cellulose, suberin, and pectins within cell walls in the cortex (red and yellow autofluorescence, Fig. 3a,b). Suberized Casparian-like strips occurred between the cells in the endodermis, and the endodermis layer remained joined to the collapsing stele during desiccation (Fig. 3c,d; Casparian-like strips are bright yellow in Fig. 3d). A parenchyma layer containing densely packed chloroplasts (chlorenchyma) lay immediately interior to the endodermis (chloroplasts exhibit red autofluorescence in Fig. 3c,d). The chlorenchyma chloroplasts autofluoresced more brightly in the resurrected state (Fig. 3d) than in the desiccated state (Fig. 3c) and less brightly in fixed tissue (Fig. 3f). The xylem conduits contained a small degree of lignin, evidenced by a weak turquoise-blue autofluorescence signal (Fig. 3c,d) and weak staining with phloroglucinol–HCl (Fig. 3e). The xylem conduits also contained pectin, which autofluoresced yellow, blending with the blue lignin signal in Fig. 3(c,d). Pectin was also evident based on the yellow fluorescence signal in Fig. 3(f). The discrete locations within the vascular cylinder for chlorenchyma and phloem tissues were clear in some samples, particularly when they were fixed before sectioning (Fig. 3e,f). Lastly, glycoproteins lined the walls of the xylem conduits, which stained pink with acid fuchsin (Fig. 3g), and this was also evident when stained samples were examined using fluorescence (Fig. 3h).

Discussion

The resurrection fern *P. triangularis* is a novel system for studying embolism formation and repair in the xylem of intact plants. We found that desiccation in *P. triangularis* dramatically differs from the drying process in desiccation-intolerant plants. In most species, cell membranes are destroyed during the desiccation process (Hoekstra *et al.*, 2001). By contrast, *P. triangularis* showed an orchestrated, reversible sequence of events during desiccation and resurrection in the stipe. Since the cortex was gas-filled in all mature stipes, all desiccation and resurrection changes in stipe tissues occurred inside the vascular cylinder. The use of microCT was valuable for elucidating the sequential dynamics of the desiccation and resurrection processes in an intact DT plant.

Conceptual model of desiccation and resurrection

We propose a biophysical model for the desiccation and resurrection processes in *P. triangularis* (Fig. 4). At frond maturity, outer cortex cells in the stipe undergo apoptosis and cellular dehydration, but these cortex tissues remain rigid, providing continued mechanical support for the vascular cylinder. Dry soil in combination with a dry atmosphere causes increased tensions on xylem water in the stele, leading to cavitation and near-complete

embolism of xylem conduits. Complete xylem embolism may initiate a controlled, consistent drying speed of the leaf tissue as well as symplastic stipe tissues (phloem and chlorenchyma). The osmotic potential of the living phloem and chlorenchyma cells is likely much lower than that of xylem sap, allowing these tissues to retain water longer than the xylem conduits, although this hypothesis has not been tested in DT ferns. The phloem and chlorenchyma shrink as the tissues lose water.

Following soil rehydration in a humid environment, the stipes rapidly rehydrate, within 24 h. First, when the roots are soaked with water, water moves through the roots into the living tissues in the stipe vascular cylinder, rehydrating the chlorenchyma and phloem. The low osmotic potential of the desiccated symplast of cells in the stipe vascular cylinder may provide the driving force for this rapid movement of water. Second, the xylem conduits refill, restoring water flow to the leaves. Three factors may contribute to embolism repair in the xylem conduits. First, capillary rise may facilitate passive refilling of xylem conduits. Second, root pressure generated by the resurrected roots (or rhizome) may push water up the xylem conduits towards the leaves. This hypothesis is supported by previous studies showing that root pressure aided refilling in DT plants (Schneider *et al.*, 2000; H. Holmlund, unpublished; but see Sherwin *et al.*, 1998). Third, densely packed chloroplasts in the chlorenchyma may be associated with high cellular sucrose concentrations, perhaps attracting water by osmosis into the stele via the roots. Desiccated steles of another DT fern species showed high sucrose concentrations (H. Holmlund, unpublished). This sucrose is likely produced during the dehydration phase and may help with stabilizing cellular structures as a compatible solute during desiccation. This is suggested in our study because we resurrected our plants at very low PPFD and production of abundant sucrose would be unlikely. It could come from hydrolyzing starch during resurrection, but we did not find starch in these tissues using I_2KI staining (data not shown). Rehydrated parenchyma cells adjacent to xylem conduits may assist with refilling, as shown previously in grapevine (Brodersen *et al.*, 2010). Furthermore, previous studies have suggested that sucrose may trigger active refilling in xylem conduits (Secchi & Zwieniecki, 2011); however, this hypothesis remains untested in DT species. Taken together, rehydration likely requires a combination of capillarity, root pressure, and metabolic activity to refill xylem conduits.

This hydraulic model for desiccation and recovery in DT ferns complements studies of embolism repair in non-DT plants. Stems (or stipes) occupy a critical position as the sole pathway for water from the roots to the leaves. Since stem xylem embolism is linked to mortality in non-DT plants, understanding mechanisms of embolism repair is timely and relevant (Pratt *et al.*, 2008; Klein *et al.*, 2018). DT ferns represent an extreme case of embolism formation, since all water is rapidly removed from the xylem conduits in the desiccated state. Few vascular plants are DT, and most DT vascular plants are small in stature (Alpert, 2006). Desiccation tolerance in vascular plants may be taxonomically limited partly because recovery from complete xylem embolism is too great a barrier to recovery from the extremely desiccated state. A better understanding of embolism repair in DT ferns may elucidate requirements for the DT trait in other vascular plants. This knowledge is relevant both to the potential development of DT crops and also to our understanding of DT evolution.

Key stipe anatomical traits

The use of light and fluorescence microscopy to reveal the chemical composition and arrangement of stipe tissues complemented our findings from microCT. For instance, autofluorescence and staining with phloroglucinol–HCl showed that the tracheid walls in these ferns are likely more flexible than the tracheids of woody plants. Pectins, glycoproteins, and reduced lignin content may provide the conduits with increased flexibility compared to the strongly lignified conduits of woody plants, preventing the conduits from breaking as the vascular cylinder shrinks during desiccation. This hypothesis is consistent with previous reports of DT leaf cell walls containing arabinan-associated pectins and arabinogalactan proteins, which are hypothesized to contribute to cell wall flexibility during desiccation (Moore *et al.*, 2006, 2008, 2013). Furthermore, this observation is consistent with previous data showing that fern tracheids have an unusually low double wall thickness to diameter ratio, likely conferring increased flexibility and low implosion resistance (Pittermann *et al.*, 2011). Additionally, a previous study has proposed that the combination of hydrophilic pectin and hydrophobic lignin increases surface wettability of xylem in maize root vessels (McCully *et al.*, 2014). Increased wettability could contribute to capillary rise in xylem conduits, but more detailed anatomical studies are required to determine if xylem conduits in *P. triangularis* show increased wettability compared to conduits in other species.

The vascular cylinder contained densely packed chloroplasts that are likely functionally important. Xylary chloroplasts have previously been linked with embolism repair and improved cavitation resistance (Schmitz *et al.*, 2012; De Baerdemaeker *et al.*, 2017), but to our knowledge, this is the first report of chloroplasts in the vascular tissue of a DT plant species. Chlorenchyma may provide a source of osmotically active molecules (sucrose), contributing to the movement of water into the chlorenchyma and phloem early in resurrection. Rehydrated parenchyma may have a role in active xylem refilling, as suggested previously (Hacke & Sperry, 2003; Brodersen *et al.*, 2010; Secchi & Zwieniecki, 2011, 2012; Secchi *et al.*, 2017). Furthermore, chloroplasts may provide energy in the form of ATP to facilitate active xylem refilling. Previous studies have suggested that energy has a role in active xylem refilling (Holbrook & Zwieniecki, 1999; Salleo *et al.*, 2004). ATP may facilitate xylem refilling by fueling the active transport of sugars into the xylem conduits to generate an osmotic gradient, but this hypothesis remains untested in DT ferns.

Light microscopy also revealed an endodermal layer with a Casparian-like strip surrounding the vascular cylinder, analogous to the endodermis found in roots. This endodermal layer is frequently found in fern stipes (Lersten, 1997; Sperry, 2003; Pittermann *et al.*, 2015). In *P. triangularis*, the endodermal layer remains attached to the shrunken vascular cylinder during desiccation (Fig. 3c,d). In a resurrection fern, this endodermal layer may act similarly to that in a root, preventing water from

leaking out of the vascular cylinder into the cortex, especially if plasmodesmata between cortex and endodermis were disrupted during development or desiccation and not repaired during rehydration. This may contribute to the lack of cortex rehydration during the resurrection process. If this is the case, the endodermis may facilitate the upward movement of water towards the leaf tissue, rather than outward movement into the cortex.

The desiccated cortex appears to provide mechanical support in both desiccated and rehydrated stipes, because desiccated fronds remain upright (H. Holmlund, pers. obs.). In hydrated plants, the cortex appears to desiccate either during development or following even slight desiccation, since all stipes of hydrated plants showed desiccated cortex except for two stipes with immature leaf tissue. This supportive function of desiccated cortex is likely facilitated by the lignin and high cellulose content of the cortex cells in their perennially desiccated state. A previous study has reported that other Cheilanthoid fern species also have lignified cortex tissue (Mahley *et al.*, 2018). Rakić *et al.* (2017) also found a gap between the vascular cylinders and the cortex in the desiccated state in DT angiosperms *Ramonda serbica* and *R. nathaliae*. However, these studies did not report findings of desiccated cortex tissue in resurrected intact plants. The phylogenetic extent of dry, lignified cortex tissue may provide insight into the evolutionary history of the DT strategy.

Whole-plant DT in other species

Since vegetative DT has evolved several times in vascular plants (Gaff & Oliver, 2013), other DT species may show contrasting mechanisms of whole-plant resurrection. For instance, *Pleopeltis polypodioides* has been shown to resurrect via specialized scales on the leaf surface (John & Hasenstein, 2017). Consequently, stipe anatomical traits may be less critical for resurrection in this species, as foliar water uptake likely plays a more significant role in the resurrection process. Additionally, several DT angiosperm species have been studied extensively, including *Myrothamnus flabellifolius, Craterostigma wilmsii,* and *Xerophyta* spp.; however, most of these studies have focused on biochemical and molecular processes (Vicré *et al.*, 2004; Farrant, 2007; Moore *et al.*, 2007; Farrant *et al.*, 2015; but see Sherwin & Farrant, 1996; Sherwin *et al.*, 1998). Desiccation and resurrection dynamics in DT angiosperm species likely differ from DT fern species due to differences in vascular anatomy, such as DT angiosperms' lack of an endodermal layer. More anatomical studies of DT fern and angiosperm species may reveal which traits are generally essential for DT.

Pentagramma triangularis may represent the resurrection dynamics in other DT fern species with a similar vascular anatomy and ecological niche. Whole-plant DT has separately evolved several times; thus, varied suites of traits likely contribute to whole-plant DT across the phylogeny. Since DT plants successfully occupy dry habitats, it will be interesting to learn what anatomical and physiological traits (or combinations of traits) are most essential for DT. Beyond increasing understanding of the DT strategy, the mechanisms of whole-plant resurrection may provide useful information about xylem refilling.

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Author contributions

All authors contributed to developing the question and experimental design. HIH, RBP and ALJ conducted microCT experiments. ALJ conducted staining and light microscopy analyses. All authors contributed to interpretation of results. HIH, JP and SDD wrote the manuscript with contributions from all authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Micro-computed tomography (MicroCT) scans of *Pentagramma triangularis* showing phloem and chlorenchyma expansion early in the resurrection process, before xylem refilling.

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