PHLOEM AS CAPACITOR - RADIAL TRANSFER OF WATER INTO XYLEM OF TREE STEMS OCCURS VIA SYMPLASTIC TRANSPORT IN RAY PARENCHYMA

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One-sentence summary: Visual evidence for the radial transfer of water from phloem into xylem supports theoretical predictions that phloem acts as a water storage capacitor in tree stems.
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ABSTRACT
The transfer of water from phloem into xylem is thought to mitigate increasing hydraulic tension in the vascular system of trees during the diel cycle of transpiration. Although a putative plant function, to date there is no direct evidence of such water transfer or the contributing pathways. Here we trace the radial flow of water from the phloem into the xylem and investigate its diel variation. Introducing a fluorescent dye (0.1% fluorescein) into the phloem water of the tree species Eucalyptus saligna Sm allowed localization of the dye in phloem and xylem tissues using confocal laser scanning microscopy. Our results show that the majority of water transferred between the two tissues is facilitated via the symplast of horizontal ray parenchyma cells. The method also permitted assessment of radial transfer of water during the diel cycle where changes in water potential gradients between phloem and xylem determine the extent and direction of radial transfer. When injected during the morning when xylem water potential rapidly declined, fluorescein was on average translocated further into mature xylem (447 μm, ±188 μm) compared to nighttime when xylem water potential was close to zero (155 μm, ±42 μm). These findings provide empirical evidence to support theoretical predictions of the role of phloem-xylem water transfer in hydraulic functioning of plants. This method enables investigation of the role of phloem tissue as a dynamic capacitor for water storage and transfer and its contribution towards maintenance of functional integrity of xylem in trees.
INTRODUCTION

Physiological and hydraulic functioning of the two long-distance transport systems in trees, xylem and phloem, have intrigued plant researchers for more than a century. Since the pioneering work of Dixon and Joly (1895; Cohesion-Tension Theory for xylem) and Münch (1930; Pressure Flow Hypothesis for phloem), the majority of studies have investigated these systems independently of each other. Although the work of Stout and Hogland (1939) as well as Biddulph and Markle (1944) laid the foundation for the physiological nexus between xylem and phloem, it is only recently that we have begun to understand the importance of the hydraulic nexus (e.g. Hölttä et al., 2006, 2009; Sevanto et al., 2011, 2014). Processes related to both nexus occur in parallel and here the term physiological nexus envelopes all metabolite exchange, including the bi-directional flow of amino acids, minerals and carbohydrates (see seminal reviews by van Bel, 1990, 2003; but see also De Schepper et al., 2013; Ferrier et al., 1975; Pfautsch et al., 2009, 2015; Wardlaw, 1974). The term hydraulic nexus refers to the function of phloem as a capacitor, where water stored in phloem moves into xylem vessels to maintain integrity of the transpiration stream (Zweifel et al., 2000 and references therein). Throughout the text we use the term phloem collectively for cells that make up transport phloem of woody plants (including sieve element/companion cell complexes, parenchyma cells, etc.), as opposed to collection and release phloem tissue, which differ in structure and function. Transport phloem is characterised by “[retention of] high hydrostatic pressure by retrieval of leaked osmotica accompanied by water flux” (sensu Patrick, 2013).

According to the Cohesion-Tension Theory, water in xylem vessels is constantly under tension and moves in a meta-stable state from roots to leaves along a hydrostatic pressure gradient. Depending on both availability of soil moisture and vapour pressure deficit of the atmosphere, this tension can exceed the cohesive forces that bind water molecules, resulting in formation of a gas void that after expanding can lead to rupture of the water column inside individual vessels (termed cavitation; Zimmermann, 1983). Once cavitated, vessels become dysfunctional and transport of water and nutrients to leaves declines. However, water stored in woody tissues of trees can be mobilized to alleviate the risk of cavitation and recent theory suggests that both water and carbohydrates from phloem may aid in reversal of vessel
embolism (i.e. air intrusion), although the evidence is indirect (e.g. Borderson et al., 2010; Nardini et al., 2011; Salleo et al., 2009).

All parts of plants have a water storage capacity (symplastic and apoplastic) and this ‘capacitance’ increases with tree size and age (Phillips et al., 2003). The ability to mobilize stored water varies according to the force required to ‘drag’ it out of storage (Holbrook, 1995). Half a century ago Reynolds (1965) highlighted the importance of the volume of internally stored water to support transpiration of trees. Since then studies have quantified the fraction of stored water in total daily transpiration for a range of tree species. This fraction varies between 2 and 20% (e.g. Tyree and Yang, 1990; Čermák et al., 2007 and references therein; Barnard et al., 2011; Pfautsch et al., 2013) and is generally smaller in angiosperms compared to gymnosperms where a maximum fraction of 50% was reported for *Pinus sylvestris* (Waring et al., 1979). Given that daily water use of large adult trees can easily reach 260-380 litres (e.g. Čermák et al., 2007; Pfautsch et al., 2011), considerable volumes of stored water must be mobilized from and restored back into capacitors on a daily basis. Remobilization of stored water can also prolong stomatal opening and thus increase carbon gain (Goldstein et al., 1998).

The volume of stored water released depends on the elasticity of the storage tissue, its connectivity to xylem vessels and the gradient of water potential ($\psi$) between the storage tissue and vessels. Cells with elastic walls represent ideal capacitors because they can change their volume as consequence of small changes in $\psi$. Thus, phloem, cambium and juvenile xylem cells are well suited for water storage and release (Yang and Tyree, 1992; Zweifel et al., 2014). The magnitude of release and refill of stored water in trees can be approximated by separately measuring the change in thickness of phloem and xylem during a diel cycle using high-precision dendrometers (e.g. Zweifel et al., 2014). Whitehead and Jarvis (1981) have calculated that around 90% of the diurnal change in stem radius can be attributed to change in water content of cambial and phloem tissues. To date it is commonly accepted that tree bark, independent of wood below, swells during the night and shrinks during the day (Zweifel et al., 2000), reflecting the water flow dynamics that characterise the dynamic exchange of water between phloem and xylem.

Phloem and xylem tissue is separated by rows of intermediary cambial cells. However, depending on the species, phloem and xylem are connected through uni- or multiseriate strands of radially aligned ray parenchyma cells, commonly termed wood
rays. These rays have been shown to be capable of symplastic water transport through plasmodesmata (Höll, 1975). Based on measurements of radial conductance, Sevanto et al. (2011) suggested that aquaporins might also be involved in radial transfer of water. Both theoretical and experimental approaches have been developed to better understand the dynamic exchange of water between xylem and phloem. Hölttä et al. (2006, 2009) developed a model based on Münch’s hypothesis and included a term that represents the hydraulic connection between the two tissue types. Through incorporating this term, model outputs suggest the occurrence of constant exchange of water between xylem and phloem along gradients of $\psi$. However, some authors suggested that changes in $\psi$ of xylem alone might be insufficient to account for observed diurnal shrinkage and swelling of bark (Sevanto et al., 2003). Along this line of argument, loading and unloading of carbohydrates in phloem tissue has been suggested to further impact radial transfer of water and associated changes in bark thickness (Mencuccini et al., 2013).

Nevertheless, to date all approaches remain indirect and the routes of water transfer between phloem and xylem have yet to be determined. Here we present a technique that enables visualisation of water transfer from phloem to xylem tissues and resolve the apoplastic and symplastic pathways and cell types. The method involves injection of an aqueous solution that contains fluorescent dye into phloem followed by analyses of woody tissues using confocal laser scanning microscopy. We assess the effectiveness of three different dyes to stain possible transfer pathways. We also introduce dye during different time intervals of the diel transpiration cycle to test the effect of predicted dynamic changes in $\psi$ between phloem and xylem on transfer processes. We hypothesized that radial transfer of water would be most pronounced during periods where conditions of the hydraulic nexus between phloem and xylem differ the most. These differences are expected during high rates of transpiration that cause a steep decline in xylem $\psi$, commonly observed during morning hours. We use leaf water potential ($\psi_L$) and high precision dendrometer measurements to identify relevant time intervals. The simultaneous assessment of leaf water potential and independent diameter fluctuation of phloem and xylem may provide empirical evidence for the role of phloem as water storage capacitor that helps in mitigating increasing tension in the transpiration stream.

**RESULTS**
Tissue anatomy

Unstained tissue samples of *Eucalyptus saligna* stems clearly show uniseriate rays that extend from phloem, through cambial tissue into xylem (Fig. 1A). The ray cells are adjacent to both sieve element/companion cell complexes and parenchyma cells in the phloem tissue (Fig 1B), while in the xylem they all traverse vessel walls (Fig. 1A, 1C). A close examination of the xylem vessels reveals that at the intersection of ray parenchyma and the vessel, inner walls of vessels are perforated with bands of large non-vestured contact pits (Fig. 1D). Where rays are absent, only a small number of vestured pits connect vessels with surrounding fibre cells. These observations confirm that the transpiration stream in xylem vessels is directly connected to phloem tissue through strands of continuous ray parenchyma.

Movement of fluorescent dyes from phloem into xylem

The three water-soluble fluorescent dyes showed clear differences in their utility in tracing the pathway from phloem into xylem tissue. When injecting fluorescein into the bark of actively transpiring trees, tissue surrounding the point of injection absorbed the fluid that contained the dye and left the injection wound empty. This documents uptake of fluorescein located in the apoplast of the phloem by sieve element/companion cell complexes and parenchyma cells. However, this absorption was locally confined to the point of injection and the tracer did not spread further in these cell types (Fig. 2A). Small amounts of fluorescein may have also been absorbed directly by ray parenchyma cells that were severed during the drilling process. More importantly, considerable amounts of fluorescein were detected in ray parenchyma leading away from the point of injection, indicating that after uptake from the apoplast, the dye was conducted radially (i.e. assuming bulk flow) in the symplast of rays. The dye clearly stained a great length of rays towards the cambium and remained in ray parenchyma, leaving the surrounding cell types unstained (Fig. 2A). Fluorescein was also detected in rays that crossed the transition from phloem into cambial tissue (Fig. 2B) and was relocated in rays and a small number of vessels in the xylem (Fig. 2C). In some tissue sections fluorescein had stained ray parenchyma as far as 800 µm into the xylem. Generally when traveling symplastically in the xylem section of rays, fluorescein was not detected past the first ray-vessel contact zone. Assessment of disks above and below the point of injection showed no signs of vertical movement of fluorescein in phloem tissue. As evidenced by control experiments that used stem
sections, which were cut from freshly felled trees prior to injection of fluorescein, no radial transport of the dye occurred when the $\psi$ in stem tissues had presumably equilibrated (see Supplemental data for detailed images). This observation is important as it demonstrates that movement of fluorescein in rays requires a gradient in $\psi$ and associated flow of water.

Unlike fluorescein, neither eosin Y nor rhodamin B was transported in the symplast of ray parenchyma cells. Eosin Y exclusively stained phloem parenchymal tissue adjacent to the point of injection (Fig. 2D). The dye appeared to slowly diffuse towards the cambium through cell walls of parenchymal tissue (see Supplemental data for more images). Rhodamin B remained mostly stationary in the area of injection, staining a few individual ray parenchymal cells, but did not stain either sieve element/companion cell complexes or phloem parenchymal tissue (Fig. 2E and see also Supplementary data). Thus, neither eosin Y nor rhodamin B appears to be an effective tracer to study symplastic or apoplastic radial transfer of water in tree stems.

Temporal variation of plant hydraulic traits and related dye-transfer

The day of concurrent leaf water potential and dendrometer measurements was mostly sunny, with air temperature (T) reaching 30 °C and photosynthetically active radiation (PAR) just below 2000 µmol m$^{-2}$ s$^{-1}$ (Fig. 3A). From morning to midday hours, vapour pressure deficit (VPD) increased from around 0.2 kPa to 2.1 kPa. Around 1400 hours a small thunderstorm was responsible for a temporary decrease in T, VPD and PAR (Fig. 3A). Once the thunderstorm had passed all weather parameters resumed their common diel course and by dusk had returned to values similar to those of the previous night.

Water potential of leaves ($\psi_L$) at pre-dawn remained stable around -0.19 MPa (±0.06, ±1 standard deviation) until 0900 hours from where it continuously declined, reaching -1.55 MPa (±0.65) at 1230 hours (Fig. 3B). Thereafter $\psi_L$ became less negative, returning close to pre-dawn values after sunset. The gradual increase in $\psi_L$ during the afternoon was briefly interrupted during the thunderstorm where $\psi_L$ rose up to -0.46 MPa (±0.18).

From midnight until 0800 hours the mean thickness of phloem and underlying cambial tissue increased by 57 µm (±7.13) for the three sampled trees (Fig. 3C). At the same time xylem tissue contracted marginally by 3 µm. During daytime, measurements of phloem thickness mirrored $\psi_L$ accurately, declining towards midday.
(marked as 1 in Fig. 3C) and increasing during the afternoon. During this time, the movement of xylem showed the exact opposite trend, albeit at a much lower rate. The total daily amplitude of phloem movement spanned more than 75 µm before reaching dimensions at 2100 hours that were equal to those at maximum expansion during the early morning. Based on measurements of leaf water potential we assumed that at this time water potential differences between xylem and phloem were minimal and any further increase of phloem thickness was mostly the result of cell growth (marked as 2 in Fig. 3C). The amplitude of movement of xylem tissue during daytime averaged 13 µm among trees.

It is noteworthy that the variation in movement of xylem tissue among the measured trees was larger when compared to that of phloem (see shading in Fig. 3C). In addition, both tissues responded immediately, and in opposite directions during the thunderstorm, providing further evidence for a temporally coupled hydraulic interconnection of phloem and xylem (Fig. 3C).

Injections of fluorescein that specifically targeted the two time windows of bark shrinkage (morning) and swelling (evening) showed visually clear and statistically significant differences (p<0.001) in the distance of migration of dye in rays (Fig. 4). For these ‘time of day’ injection experiments, measurements of distances the dye had moved were pooled from multiple independent sample specimens (Table 1). When injecting dye before 0800 hours into phloem and collecting tissue samples around midday, the dye had moved on average 447 µm (±188 µm) into the mature xylem where it often reached the first ray-vessel contact zone (Fig. 4A). The distance that dye had been translocated in rays varied from 127 to 807 µm, indicating pronounced variability among individual rays. In contrast, when injecting the dye at dusk and collecting tissue samples at pre-dawn the following morning, the dye inside ray parenchyma had migrated mostly into the zone of cambial and juvenile xylem tissue (Fig. 4B). Although this incubation period was three times longer (12 hours) compared to experiments during the morning hours (4 hours) fluorescein had moved on average just 155 µm (±42 µm). Injections at dusk produced relatively uniform staining patterns in rays. Measured transfer distances ranged between 68 and 254 µm. In none of the examined samples did the stain reach mature xylem and conducting vessels.

DISCUSSION
The combination of experimental (in- and ex-situ) and analytical techniques applied here provides direct evidence for the radial transfer of water from phloem to xylem tissue in tree stems as predicted from theory (e.g. Hölttä et al., 2006; Lacointe and Minchin, 2008) and empirical but indirect measurements (e.g. De Schepper and Steppe, 2010; Sevanto et al., 2011) of the hydraulic nexus of phloem and xylem. This transfer of water is predominantly facilitated through the symplast in uniseriate ray parenchyma cells, driven by transpiration and associated gradients in $\psi$ between the two tissue types. We do acknowledge that by injecting the dye we can not separate diffusion from bulk flow of fluorescein in the symplast of ray parenchyma and the final distance the dye reached in rays is the result of both movements. However, transfer of dye did not occur due to diffusion as documented by the results of our ex-situ control experiment using isolated, cut stem sections. Hence this transfer must be mediated by bulk flow of both water and dye. It is reasonable to speculate that water and stain entered the rays using different mechanisms (e.g. aquaporins vs. plasmodesmata). Their exact routes of uptake remain to be confirmed experimentally. The initial uptake of water containing the tracer must have taken place from the apoplast into the symplast of ray parenchyma cells. This indicates that $\psi$ is slightly lower in the symplast of these cells compared to the apoplast and other cell types present in the phloem. However, the fact that once the tracer was inside the symplast of ray parenchyma cells and no staining of any surrounding cells was observed, underpins the dominance and tight regulation of this transport pathway. Although we did not detect any fluorescence in the apoplastic space, we cannot entirely exclude the possibility that some water molecules of the water-tracer solution followed this pathway. Once taken up into living ray cells, the tracer moved inwards by diffusion and/or bulk flow passing through plasmodesmata that connect ray cells. The importance and involvement of the plasmodesmatal pathway in translocation of organic and inorganic substances has long been known (e.g. Sauter and Kloth, 1986). As fluorescein cannot diffuse through plasma membranes (and thus is a tracer specific to symplastic pathways), the tracer was retained in the region of contact pits where only small traces of the dye may have leaked out into the adjacent xylem vessel. Our results provide support for a functional role of water transfer in phloem during periods of increasing tension in the xylem (morning) compared to times where differences of $\psi$ in xylem and phloem are considerably smaller (night) or absent (ex-situ controls). In addition, movement of water necessarily implies differences in the $\psi$
gradient between phloem and xylem, suggesting a greater complexity in internal water transfer in trees. Once more, our ex-situ control experiment – where equilibrium of $\psi$ in phloem and xylem can be assumed – confirms that radial transfer occurs once a difference in $\psi$ is present. We have found no traces of the dye in rays when the transpiration stream had been disrupted, not even close to the point of injection and regardless of the incubation time.

Our results highlight the function of phloem tissue as potential contributor to stem water storage and thus capacitance in trees as reported by Zweifel et al. (2000), Pfautsch et al. (2011) and others. Together with the excellent work of Sokolowska and Zagórska-Marek (2012), that documents radial transfer of water from xylem to cambial cells, we now have conclusive visual evidence for the occurrence of bi-directional flow of water between phloem and xylem. Their and our study used fluorescent tracers. Thus, fluorescein-based dyes appear highly effective for use in studies of the hydraulic nexus. While fluorescein has been shown to enter ray parenchyma cells when infusing angiosperm xylem vessels with a dye solution ex-situ (Cirelli et al. 2008; Sokolowska and Zagórska-Marek, 2012), our method is suited for in-situ applications, where naturally occurring changes in osmotic and hydraulic pressures facilitate distribution of the dye and tracing of symplastic pathways.

The fast and highly efficient response to injuries observed in phloem tissue (see references in Knoblauch and Mullendore, 2013) works to the advantage of our method. Sieve elements contain large quantities of P-protein that is mobilized immediately after an injury to sieve element/companion cell complexes, clogging sieve plates and closing the wound by formation of callose tissue (Knoblauch and van Bel, 1998). This reaction is seen as an adaptation to the pressurized state where uncontained wounds could cause extensive leaking of transported assimilates (van Bel et al., 2002). We did not detect any fluorescence in tissues sampled a few cm above or below the point of injection and attribute this result the to effective clogging of sieve plates by P-protein and possibly also by dislodgement of small cellular structures during drilling of injection holes (Knoblauch and Mullendore, 2013). We thus can exclude any appreciable loss of the injected tracer due to axial transport in the phloem.

Our study showed that not every water-soluble dye is suitable to trace movement of water in the living tissue of phloem, particularly the loading from the apoplast and symplastic transport in ray parenchyma cells. The molecular weight of sodium fluorescein is 376 g mol$^{-1}$, similar to that of sucrose (342 g mol$^{-1}$). Sucrose is the
primary form for transport of carbon in phloem of most plants and membrane-bound shuttles for this molecule are in high abundance (van Bel and Thompson, 2013). Hence using a fluorescent dye of comparable molecular weight is perhaps increasing chances for staining of symplastic pathways. Contrary to fluorescein, movement of eosin Y, and more so that of rhodamin B in phloem and rays was very limited. The molecular weight of these two dyes is substantially greater compared to fluorescein or sucrose (eosin Y: 691.85 g mol⁻¹; rhodamin B: 479.02 g mol⁻¹). We acknowledge that other factors, such as polarity or the affinity to chelate (Boens et al., 2007) could further influence the effectiveness of a dye to trace either apo- or symplastic pathways in woody tissues.

Observed differences in tracer localization among cell types however, raises an interesting question. Is the efficiency of symplastic transport of water in rays superior to that of the apoplast (i.e. cell walls, intercellular spaces)? Fluorescein, the smaller molecule, moved much further when transported in ray parenchyma compared to eosin Y, the larger molecule, diffusing in the apoplast. Also other dye experiments have found that the symplast is favoured over the apoplast in transport routes (Sano et al., 2005; Sokolowska and Zagórska-Marek, 2012). As shown in the scanning electron micrographs, flow of large quantities of water could be accommodated through the wide and numerous contact pits that connect rays with vessels. Such connections have been described for many woody species (Schoot and van Bel, 1989) and are not present where rays connect to surrounding phloem cells. These connections are yet to be characterised in detail (van Bel, 1990; Patrick, 2013). It could be speculated that if rays are well connected at both ends of the pathway, a lower resistance to symplastic flow and good connection to xylem vessels would make this pathway superior to that of the apoplast. Experiments using dyes with different properties to fluorescein (e.g. Texas red that is not taken up by cells; isotopic tracers like deuteriated water) are needed to improve our understanding of apo- and symplastic pathways and their quantitative importance.

Further insight into plant hydraulic functioning arises when tracing these transport pathways. Here we showed that fluorescein did not migrate beyond the point where the individual ray intersected the very first vessel. Our anatomical observations support the conclusion that the bulk of the water transferred from phloem to xylem was incorporated into the transpiration stream, as fluorescein never passed the most
recently formed vessels in which \( \psi \) would be more negative compared to that in adjacent ray, fibre or parenchymal cells. Differences in the distance that fluorescein had migrated following injections during the morning and evenings are in agreement with the theoretical work of Hölttä et al. (2009). Water potential gradients are naturally greatest during periods of high transpiration, reflected in our measurements of declining \( \psi_L \) and associated contraction of phloem tissue during the morning hours. These conditions resulted in translocation of the dye farther into mature xylem. In contrast, the dye was only translocated into the region of cambial and juvenile xylem tissue during the night when transpiration is generally slow. The efflux of water from phloem during times of low transpiration is mostly a combination of unloading of carbohydrates to fuel cell expansion in the cambial zone and Münch counterflow (Hölttä et al., 2006). During our control experiments where transpiration had ceased altogether in cut stem sections, the dye did not enter the rays at all.

**CONCLUSIONS**

By direct injection of fluorescein into phloem tissue of *Eucalyptus saligna* we have provided visual evidence that radial transfer of water from phloem into xylem predominately occurs via the symplast of ray parenchyma cells. The distance of dye migration into xylem differed between day and night as a consequence of differences in water potential between the tissue types. Results presented here show that injections of fluorescent dyes, together with measurements that capture hydraulic functioning of trees can successfully be used to improve our understanding of whole-tree physiology. Although an important first step, our findings provide guidance for future research. Comparisons of apo- and symplastic pathways as well as quantification and timing of radial transfer of water appear to be the most pressing issues.

**MATERIAL & METHODS**

**Plant Material**

We used more than 30 stems of *Eucalyptus saligna* Sm located on the premises of the Hawkesbury Forest Experiment, 60 km northwest of Sydney, Australia (25 m a.s.l; 33° 36' 40" S, 150° 44' 26.5" E). The trees were between 6 and 8 meters tall and had regenerated from rootstocks that were row-planted in a plantation. Diameter of the
stems ranged between 5.1 and 7.2 cm and thickness of the smooth gum-type bark was 4-5 mm. Climate of the area is temperate sub-humid with an average annual temperature of 17 °C. Mean annual precipitation at the site is around 800 mm, with wet summers and relatively dry winters. We measured leaf water potential and dynamic changes of bark and sapwood thickness of three trees on 5 March 2014. Environmental conditions of that day were recorded on site by a weather station (CR3000, Campbell Scientific, Garbutt, Australia), including air temperature (T, °C), relative humidity (rH, %; HMP45C, Vaisala, Helsinki, Finland) and photosynthetically active radiation (PAR, µmol m⁻² s⁻¹; SQ-110, Apogee Instruments Inc., Logan, Utah, USA). Vapour pressure deficit (VPD, kPa) was calculated using T and rH according to Snyder and Shaw (1984). All dye injections and associated sample collections and analyses took place between 7 March and 16 May 2014, during periods of generally sunny conditions.

**Leaf water potential**

Leaf water potential (\(\psi_L\)) is a good indicator for hydraulic tension in xylem and measurements of \(\psi_L\) were used as surrogate to characterize the daily evolution of tension in xylem water of stems. For this purpose three leaves were randomly sampled from the upper canopy of three *E. saligna* trees (n = 9) at hourly intervals between pre-dawn and post-dusk (0600 – 1900 hours). After excision, leaves were placed in a darkened bag that contained a moist paper towel to prevent further loss of water. Bags were immediately transferred to a nearby research laboratory where \(\psi_L\) was measured using a pressure chamber (1505D, PMS Instrument Company, Albany, Oregon, USA).

**Dendrometer measurements**

The diurnal variation in diameter of phloem and xylem was recorded using high-precision point dendrometers (ZN11-O100-2WP, Natkon, Switzerland). A week prior to measurements of \(\psi_L\) pairs of dendrometers were installed 70 cm above the ground on three stems. A 3×3 cm wide bark window was removed and the exposed sapwood was washed with distilled water to remove cambial cells. The transducer of the dendrometer was placed onto the sapwood and the window was sealed using silicon grease. The transducer of a second dendrometer was placed onto the bark surface. Both dendrometers were mounted onto a carbon fiber ring that was affixed to the stem by drilling screws into the sapwood. This experimental set-up is described in...
detail in Zweifel et al. (2014). Data were logged (CR1000, Campbell Scientific Inc., Logan, Utah, USA) at 5-minute intervals. We subtracted values measured on sapwood from those measured on bark to obtain values that represent diameter variation of phloem and underlying cambial cells through time.

**Fluorescent dyes**

Fluorescein derivatives in aqueous solution have been shown to move well in sieve tubes of the phloem and vessels and ray parenchyma of the xylem (e.g. Grignon et al., 1996; Cirelli et al. 2008; Sokolowska and Zagórska-Marek, 2012), but care has to be taken, as emission characteristics may be similar to those of woody tissues (Palmquist, 1938). The present study used a 0.1% solution of fluorescein (sodium fluorescein, Sigma Aldrich, Sydney, Australia) that exceeds the autofluorescence of woody tissues, emitting maximally at 520nm and exceeding the more blue-shifted autofluorescent emissions of woody tissues. In addition, the mobility of two other fluorescent dyes, namely eosin Y and rhodamin B, was also assessed using solutions at 0.1 % strength. Eosin has been shown to trace uptake of water through twigs in *Picea abies* where it was detected in wood and the bark (Katz *et al.*, 1989). A rhodamin dye has successfully been used to trace water movement in leaf veins of maize plants (Canny and McCully, 1986).

**Dye injections and sample collection**

Small holes were drilled in tangential direction into the phloem tissue with help of hypodermic needles (outer diameter 0.64 mm) connected to 5 ml syringes. Each hole was filled with fluorescent dye using minimal force, ensuring that at least 100 µl of the solution was released into each hole. During initial experiments dyes were allowed to disperse for two days. After assessment of these injections with three dyes, it became clear that only fluorescein could be detected in xylem ray parenchyma. For this reason fluorescein was used in subsequent experiments to examine diurnal radial movement of water via shorter time intervals between injection and sample collection ('morning’ = 0800 to 1200 hours; ‘night’ = 1830 to 0630 hours the following day). For each time interval we used six tree stems. Fluorescein was injected at two opposing positions into the phloem tissue at the basal segment of the stem. In addition to these *in-situ* injections we also included *ex-situ* injections as a control experiment. For this experiment we excised basal stem sections (30-35 cm length) of ten trees
between 0700 and 0800 hours. Immediately after excision both cut surfaces were sealed to prevent any evaporative water loss and associated movement of water. The sections were left to rest for one hour before fluorescein was injected following the identical procedure described above. Stem sections were positioned upright and left for a minimum of 6 hours under ambient conditions in the laboratory.

A total of 42 stem disks (20 mm thickness) containing the point of injection were collected using a pruning saw. Adjacent disks from above and below the point of injection (n = 84) were also cut to assess possible vertical movement of the dye. Promptly after cutting the discs, samples were wrapped in moist paper towels, placed in a plastic bag and transferred to the lab where the region of dye injection was extracted using a hammer and chisel, separating wood samples into stained and non-stained material. Non-stained material was used to prepare free hand and microtomed sections. The same material was used for scanning electron microscopy, for which all sides of sample blocks were trimmed using a microtome to provide clean and smooth surfaces. Samples from the injection area were cut into cubes of approximately 8-10 mm size and the radial surface of one side of such blocks was trimmed with a razor blade to provide a clean and smooth surface for scanning laser confocal microscopy. Extra care was taken during all processing steps that the connection between bark and sapwood remained intact. All sample blocks were kept at 4 °C and processed within 24 hours.

**Scanning electron and optical microscopy**

Fresh sample blocks were mounted on aluminum stubs with double carbon adhesive tape allowing imaging of radial and transversal planes. A scanning electron microscope (6510LV, JEOL, Peabody, USA) operated in low vacuum mode at a vacuum chamber pressure of 50Pa and 20kV accelerating voltage and 15mm working distance was used for imaging the surface of the samples.

Free hand sections of intact bark-phloem-cambium-sapwood sequences were prepared using a razor blade and transferred onto glass slides. Radial sections (25 µm thickness) of sapwood were prepared from 2 cm cubes using a sliding microscope (Leica RM2255, Leica Microsystems, Wetzlar, Germany). All microtomed sections were stained with safranin and washed clean before embedding on glass slides with cover glass. Digital images of stained (10X) and unstained (4X) samples were taken using a transmission light microscope (Leica SM2010 R, Leica Microsystems,
Germany) equipped with a high-resolution (12 Megapixel) digital camera (Leica DFC 500, Leica Microsystems, Germany).

**Laser scanning confocal microscopy**

Samples stained with fluorescent dyes were analysed using an inverted Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany) suited for live-cell microscopy. This microscope uses the Acousto Optical Beam Splitter (AOBS®) for excitation–emission separation, making it particularly suited for imaging and separating multiple fluorophores without the need to use specific filters or beam-splitters. Confocal imaging of the phloem and the xylem components was by excitation with the 405 nm diode laser to detect autofluorescence at 410-470 nm. A 488 nm excitation line of the Argon laser was used for the detection of fluorescein and eosin, with the detection bandwidth set at 500-560 nm. The DPSS 561 nm laser was used for the excitation of samples stained with rhodamin and detection was set at 570-630 nm. Chlorophyll was imaged with an emission channel set at 670-700 nm. Images of the different tissues of the sample were collected using a confocal zoom magnification setting of 10X. Scanning 3-7 frames for each image achieved reduction of the signal-to-noise ratio. Serial optical sections in xyz mode were collected to image samples in three dimensions, from surface to the required depths. The z series from the image stacks were reconstructed into 3D views using Leica LAS software. Microspectral imaging was done with the xyl scan mode using various laser lines for excitation of fluorophores, wood and chlorophyll autofluorescence.

We extensively tested for spectral separation of tissue and chloroplast autofluorescence and fluorescence emission of the dyes and found good separation between tissues and compounds (Fig. 2F). Full details of the spectral scans shown as composite images in Fig. 2 A, D and E are provided as supplementary materials.

The distance that fluorescein had travelled in individual rays during the morning and night was measured manually using the Leica Application Software ‘Advanced Fluorescence’ software (V4.2, Leica Microsystems). The starting point for these measurements was at the boundary of phloem and cambial cells. We measured only rays showing clear evidence of fluorescein for at least 30 µm past this border. Shapiro-Wilk’s test confirmed a normal distribution of the measured distances of dye movement in the two sample populations. A statistical test of differences in measurements between the morning and night injection treatments was conducted.
using the Kruskal-Wallis test for non-homogeneous sample sizes between populations.

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The authors thank Liz Kabanoff for her help in selecting the dyes and the site managers at the Hawkesbury Forest Experiment, Craig Barton and Burhan Ahmiji for provision of plant material and practical help in the field. We would like to acknowledge the help with SEM imaging at the Advanced Materials Characterisation Facility at the University of Western Sydney by Richard Wuhrer. Comments on the draft manuscript by Maurizio Mencuccini and two anonymous reviewers were highly appreciated.

The authors declare no conflict of interest.
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FIGURE LEGENDS

Figure 1: Micrographs showing anatomical structures in woody tissues of *Eucalyptus saligna*. A, Intact free-hand transverse section showing phloem (right), cambium (c) and mature xylem tissue (left), including xylem parenchyma (xp) and vessels (v). Intact wood rays (r) traverse from phloem through the cambium into the xylem. Scale 250 µm. B, Electron micrograph showing the transverse view of phloem, where rays (r) extend through phloem parenchyma (pp) and sieve element/companion cell complexes (se/cc). Numerous calcium oxalate crystals are visible. Scale 100 µm. C, Detailed transverse view of xylem vessels bordered by uniseriate rays, surrounded by parenchymal tissue (xp). Scale 100 µm. D, Electron micrograph (radial plane) depicting vertical wood fibres (f) and the broad bands of non-vestured contact pits (cp) connecting the vessel (v) with ray cells. Scale 100 µm.

Figure 2: Staining characteristics (transverse view) several hours after injection of fluorescent dyes into phloem tissue of *Eucalyptus saligna* and emission spectra of tissues and dyes. Arrows indicate direction of water flow from phloem into xylem tissue. A, Injection wound (iw) of the needle used to introduce fluorescein in phloem tissue and migration of the dye in ray parenchyma. B, Transition of fluorescein from phloem towards xylem tissue. C, Fluorescein passing cambial tissue, entering mature xylem and staining vessels. D, Injection wound where eosin Y was introduced and spread of the dye into phloem parenchymal tissue. E, Injection wound where rhodamin B was introduced, showing that the dye remained mostly stationary. F, Relative fluorescence emission spectra of woody tissues (phloem parenchyma and sapwood ➀, auto-fluorescence), fluorescein ➁, eosin Y ➂, rhodamin B ➃ and chlorophyll ➄, auto-fluorescence). Micrographs are composite images of bandwidths scanned for woody tissue, fluorescent dye and chlorophyll. Individual images of all channels are provided as supplementary material. Scale 200 µm in all panels.

Figure 3: Environmental and physiological conditions during a diel time course (5 March 2014). A, Photosynthetically active radiation (PAR) and vapour pressure deficit (VPD). B, Diurnal course of leaf water potential ($\psi_L$) collected from the upper canopy of three *Eucalyptus saligna* trees (n = 9 leaves per time point); error bars depict ±1 SD. C, Differential of the movement in phloem (black line) and xylem
tissue (blue line) in basipetal stem sections of *E. saligna* (n = 3 trees, shaded areas represent ±1 SD) zeroed according to measurements recorded at 0000 hours; numbers and arrows indicate the phase of rapid shrinkage of phloem thickness during morning hours (1) and growth during the night (2); the grey dotted line marks the point where phloem rehydration is complete and wood growth occurs (see text for details).

**Figure 4:** Contrasting day vs. nighttime progression of fluorescein (transverse view) in ray parenchyma of *Eucalyptus saligna*. Dashed lines indicate borders between tissue types, where p = phloem, c = cambial zone and juvenile xylem, x = mature xylem. Yellow bars show the maximum distance that fluorescein had progressed. A, Injection of the dye at 0800 hours, tissue harvest at 1200 hours. B, Injection of the dye at 1900 hours, tissue harvest prior to 0700 hours of the following morning. Scale 200 µm in both panels.
Table I: Details of in-situ dye injections into the phloem tissue of *Eucalyptus saligna* tree stems during two different time intervals.

<table>
<thead>
<tr>
<th>Time of injection</th>
<th>Number of trees</th>
<th>Total injections</th>
<th>Sample specimens(^1)</th>
<th>Number of rays containing fluorescein measured</th>
<th>Distance of fluorescein spread from phloem-cambium boundary (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning</td>
<td>6</td>
<td>12</td>
<td>9</td>
<td>49</td>
<td>447.1 (188.4)</td>
</tr>
<tr>
<td>Evening</td>
<td>6</td>
<td>12</td>
<td>8</td>
<td>44</td>
<td>155.1 (42.0)</td>
</tr>
</tbody>
</table>

\(^1\)Number of sample specimens of intact bark-phloem-cambium-sapwood sequences at the point of injection used for image analysis. Data shown are mean (SD)
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