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Review

The functional role of xylem parenchyma cells and aquaporins during recovery from severe water stress

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ABSTRACT

Xylem parenchyma cells [vessel associated cells (VACs)] constitute a significant fraction of the xylem in woody plants. These cells are often closely connected with xylem vessels or tracheids via simple pores (remnants of plasmodesmata fields). The close contact and biological activity of VACs during times of severe water stress and recovery from stress suggest that they are involved in the maintenance of xylem transport capacity and responsible for the restoration of vessel/tracheid functionality following embolism events. As recovery from embolism requires the transport of water across xylem parenchyma cell membranes, an understanding of stem-specific aquaporin expression patterns, localization and activity is a crucial part of any biological model dealing with embolism recovery processes in woody plants. In this review, we provide a short overview of xylem parenchyma cell biology with a special focus on aquaporins. In particular we address their distributions and activity during the development of drought stress, during the formation of embolism and the subsequent recovery from stress that may result in refilling. Complemented by the current biological model of parenchyma cell function during recovery from stress, this overview highlights recent breakthroughs on the unique ability of long-lived perennial plants to undergo cycles of embolism-recovery related to drought/rewetting or freeze/thaw events.

INTRODUCTION

Trees regularly cope with environmental stressors, many of which have been exacerbated recently by climatic alterations across the planet. Over the years, the plant physiology community has focused increasing attention on drought stress, which is known to induce a complex network of physiological effects including the xylem embolism formation/recovery cycle. The way in which plants sense and recover from embolism is a matter of particular research interest because of its relevance to their intrinsic ability to handle the transport of water under tension. However, while embolism formation is a purely physical phenomenon related to xylem chemistry and morphology, xylem refilling requires the generation of water flow against a pressure gradient. Thus, recovery from embolism can only be understood through consideration of biological activities capable of providing the energy and water needed to restore hydraulic conductivity. In recent years, significant efforts have been made to gain knowledge on this debated process, but a comprehensive understanding of the biological processes involved in xylem recovery from embolism in tree stems remains elusive.

This review explores recent research advances in woody plant embolism repair theories, which take into account the biological processes occurring at stem and cellular levels.

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XYLEM MORPHOLOGY AND PARENCHYMA CELLS IN WOODY PLANTS

Long-distance water transport in vascular plants occurs through a network of conduits built from nonliving cells (xylem) along the stem, branches, twigs, petioles and leaf veins that connect roots to leaf mesophyll cells (Sperry *et al.*, 2003, Tyree & Zimmermann, 2002). The xylem represents about 99% of the entire length of the water transport pathway from roots to photosynthetic tissues, and the remaining 1% consists of a few millimetres of extra-vascular pathways that water follows when moving from the root surface to the stele and from leaf minor veins to evaporation sites (Cruiziat *et al.*, 2002, Nardini *et al.*, 2011b).

Xylem conduits are long hollow tubes with finite dimensions, dead at maturity (Carlquist, 2015, Comstock & Sperry, 2000). They are characterized by thick and lignified walls capable of sustaining large negative pressures (Hacke *et al.*, 2001a, Pittermann *et al.*, 2006). Gymnosperms possess xylem conduits (tracheids) that are uniform in shape and length and connected to each other through small openings in the secondary cell walls (bordered pits). Most conifers have a pit membrane structure with a porous margo and central torus assembly (Zimmermann, 1983, Choat *et al.*, 2008, Pittermann *et al.*, 2005). In Angiosperms, the water transport conduits are more specialized vessels consisting of drum-shaped cells (vessel elements). The end walls between vessels are open (perforation plates) with elements stacked end on end to form long tubes extending over several centimetres or even metres. Water transfer from vessel to vessel occurs through bordered pit-fields, which consist of multiple small openings separating adjacent vessels with thin cellulose/pectin membranes (Holbrook & Zwieniecki, 2005, Tyree & Zimmermann, 2002).

Tracheid and vessel elements are the key structural components of long-distance water transport, but the xylem as a

whole is not made of solely dead conduits. The bulk of secondary xylem (functional xylem) contains, besides fibres, an interconnected network of living cells that links heartwood (non-functional xylem compartmentalized within the stem) and phloem (stem parenchyma cells). These parenchyma cells usually have thin walls and are rectangular or square in shape (Morris *et al.*, 2016). They are formed by fusiform and ray initials of the vascular cambium and are oriented both axially and radially. The living parenchyma cells can represent a large component of the tissue volume and the abundance of those varies across environments, plants organs and species (Holbrook & Zwieniecki, 2005, Spicer, 2014). Stems have fewer, smaller and tighter ray parenchyma cells than the roots (Denne & Gasson, 2008, Morris *et al.*, 2016, Pratt *et al.*, 2007). Moreover, recent meta-analysis has found significant differences between volumes of ray and axial parenchyma across climatic zones with higher volumes observed in tropical trees and lower volumes in trees and shrubs growing in temperate and subtropical areas (Morris *et al.*, 2016). Conifers were found to have far less radial and axial parenchyma in xylem than angiosperms. Lianas and stem succulents represent some of the most parenchyma-rich stems in the plant world (Spicer, 2014), a pattern that reflects an increased demand for mechanical elasticity in climbing plants and the need for water storage in succulents (Brandes & Barros, 2008, Carlquist, 2015, Chapotin *et al.*, 2006, DeSmidt, 1922).

It is assumed that living parenchyma cells play many important functional roles. However, specific functions are often derived indirectly as the location of these cells makes them difficult to study. Among these functions, the loading/unloading of solutes into/from the transpiration stream (De Boer & Volkov, 2003) and the storage and transport of carbohydrates as soluble sugars, starch and/or lipids are most often considered (Bucci *et al.*, 2003, De Boer & Volkov, 2003, Salleo *et al.*, 2004, Secchi *et al.*, 2011, Spicer, 2014, Zwieniecki & Holbrook, 2009). In addition, they may play a significant role in defense against pathogens by preventing their lateral and axial spread (Deflorio *et al.*, 2008, Morris & Jansen, 2016) and accumulating anti-microbial compounds. Some evidence of their involvement in mechanical support is also reported (attributed mostly to ray parenchyma) (Arbellay *et al.*, 2012, Reiterer *et al.*, 2002).

Recent studies point to the role of living stem parenchyma cells pathways between mature xylem and phloem, as xylem conduits are both physically and functionally associated with living phloem. Physical association is derived from a single cambium initial that produces both xylem and phloem derivatives (Larson, 1994); thus, the radially oriented parenchyma cells grouped together in rays extend from xylem to phloem. This functional association is demonstrated by several examples of the interchange of water and solutes between xylem and phloem (Metzner *et al.*, 2010, Nardini *et al.*, 2011b, Schneider *et al.*, 1994, Vanbel, 1990, Wang *et al.*, 1997).

Additionally, parenchyma cells are shown to be involved in water storage (as confirmed by the high amount of ray and axial parenchyma in stem succulents) and xylem hydraulic capacitance (Barnard *et al.*, 2013, Holbrook *et al.*, 2002, Pfautsch *et al.*, 2015, Salleo *et al.*, 2004). Therefore, a greater amount of axial and radial parenchyma cells in wood may confer higher stem hydraulic capacitance. These properties – (1) facilitation of transport between phloem and xylem, (2) energy and osmotic storage capacity, and (3) water storage capacity – were the basis of the recently proposed role of stem parenchyma cells as a crucial component in the maintenance of xylem transport capacity and embolism removal (refilling) even under small negative tensions (Nardini *et al.*, 2011a, Nardini *et al.*, 2011b, Salleo *et al.*, 2004, Secchi & Zwieniecki, 2011, Zwieniecki & Holbrook, 2009). Specifics of the biology behind and the role of parenchyma cells in embolism-recovery are detailed in this review.

Although there is no single confirmed theory explaining the dynamics of embolism repair in all vascular plants, it is worthwhile noting that compared with angiosperms, gymnosperms tend to have little parenchyma in their wood. If we assume that the ability to rapidly repair embolisms relies on the presence of nearby parenchyma cells, this may explain the long length of conifer embolism recovery time (days or months) and their need for a larger safety margin when compared with angiosperms experiencing comparable levels of embolism (Johnson *et al.*, 2012; Johnson *et al.*, 2012). It is also proposed that conifers may utilize an entirely different approach to embolism recovery that depends on the tree's ability to restore functional water potentials when exposed to fog or snow (Earles *et al.*, 2016, Mayr *et al.*, 2014). In this review, we will focus on the biology of parenchyma cells in angiosperm species and discuss their biological role in xylem recovery from severe water stress.

PARENCHYMA FUNCTION IN XYLEM EMBOLISM REPAIR

Water transport in the xylem is a purely physical process driven by a difference in water pressure. Water transport through a network of dead cellular conduits occurrs under negative pressures (tension). Because the apoplastic water column in the xylem is under tension, it is considered to be in a metastable state (Stroock *et al.*, 2014) and at risk of cavitation. Although the tensions experienced by trees are far less than the tensions required to cause homogeneous cavitation, they may be large enough to trigger cavitation from seeding sites – like the micron or submicron-sized air pockets present in the vessel crevices (Tyree & Sperry, 1989). Expansion of these gas bubbles results in the formation of embolisms that can quickly spread through an entire vessel. Further spread of embolism from vessel to vessel may occur via the penetration of air bubbles through bordered pit membranes (Brodersen *et al.*, 2013). Embolism formation is a purely physical process (Brenner, 1995, Tyree & Zimmermann, 2002) related to the degree of tension in the xylem, the chemical properties of water, the thermal environment and the physical properties of the xylem (Hacke *et al.*, 2001b, Holbrook & Zwieniecki, 1999, Stiller & Sperry, 2002, Tyree & Zimmermann, 2002). The presence of an embolism disrupts the plant's water continuum by reducing xylem transport capacity and causing short and long-term effects on plant functions. Effects include stomatal closure, reductions in the rate of photosynthesis, reductions of growth,

loss of production and even plant death (Brodribb & Cochard, 2009, Sperry *et al*., 1998).

As the prolonged presence of embolisms is a threat for plant survival, species have evolved several strategies to prevent and/or alleviate the effects of hydraulic failure and restore xylem transport functionality. These include anatomical xylem adaptation (specialized pit membrane structures, stem sectoriality and wood density), the shedding of leaves or small branches (shrubs) to lower evaporative demand and growing new vessels to replace nonfunctional conduits (Zwieniecki & Secchi, 2015). These processes are very slow and necessitate prolonged relief from water stress/transpirational demand. We might consider these long-term strategies non-competitive. However, some species growing in competitive environments demonstrate the evolution of active physiological strategies that lead to the quick recovery of xylem hydraulic functionality. These strategies include (1) the generation of positive root pressure when the soil is fully saturated (often found only in small herbaceous and smaller woody plants) (Cochard *et al*., 1994, Ewers *et al*., 1997, Yang *et al*., 2012); (2) access to external water sources (rain, fog or snow) in order to facilitate water uptake and water flow into the xylem through leaves, buds and/or bark, a strategy adopted principally by coniferous species (Earles *et al*., 2016, Laur & Hacke, 2014, Mayr *et al*., 2014); and (3) cellular activities of living xylem parenchyma cells, resulting in fast (minutes to hours) localized embolism removal in woody plants (observed primarily in angiosperm species) (Brodersen & McElrone, 2013, Brodersen *et al*., 2010, Nardini *et al*., 2011b, Salleo *et al*., 2004, Secchi & Zwieniecki, 2012), which may even occur during the presence of active transpiration (Holbrook & Zwieniecki, 1999, Trifilo' *et al*., 2003). The presence of great tension most likely precludes the occurrence of xylem refilling, but recovery has been reported under low-tension levels. Reconciling the presence of tension with embolism recovery has been proved difficult to understand, and only recently *in vivo* imaging has suggested the ability of plants to refill embolized vessels in situations with very low tensions (below 0.5 MPa) (Brodersen *et al*., 2010, Clearwater & Goldstein, 2005, Knipfer *et al*., 2015, Zwieniecki *et al*., 2013). A recent work using X-ray micro CT analysis has shown that in grapevine refilling can occur without presence of root pressure, and it is osmotically driven against low negative water potential (Knipfer *et al*., 2016). However, despite scientific efforts (Nardini *et al*., 2011a, Salleo *et al*., 1996, Secchi & Zwieniecki, 2010, Zwieniecki & Holbrook, 2009), the biological mechanisms responsible for embolism recovery under low negative pressure are not resolved beyond the general statement that living cells are involved in the recovery process.

Although embolism formation is a physical process, its removal requires that empty vessels fill with water against existing energy gradients as the bulk of water in the xylem remains under tension. In this scenario, recovery from embolism cannot happen spontaneously and necessitates (1) some physiological activities in the xylem to maintain or restore transport function (promoting water flow into empty conduits) and (2) the involvement of living parenchyma cells able to perform physiological activities during the recovery process. Existing models of xylem repair suggest that living parenchyma cells, adjacent to the xylem vessels, are at the forefront of the refilling process (Salleo *et al*., 2004). This assumption is supported by the observations of inhibition or reduction of refilling in the case of either physical damage to phloem transport or the metabolic inhibition of living cells in stems (Bucci *et al*., 2003, Salleo *et al*., 2004, Zwieniecki *et al*., 2004). It was concluded that phloem might be involved in supplying the energy to sustain xylem recovery (Bucci *et al*., 2003, Salleo *et al*., 2004), and since there is a physical separation of phloem from xylem conduits, living parenchyma cells can function as bridges that allow solutes (and water) to flow from phloem to embolized conduits (Nardini *et al*., 2011a). This pathway may involve multiple crossings of cellular membranes, thus being mediated by the activity of water channels (aquaporins), sugar transporters and plasmodesmata.

Xylem parenchyma cells in contact with xylem conduits are assumed to simultaneously generate the energy gradient (deposition of solutes in the form of sugars, ions or a combination of both) that allows water to flow into empty vessels/tracheids, and supply water for refilling (Zwieniecki & Holbrook, 2009). Regarding the first task, the initial research focus has been on finding the source of osmoticum through analysis of carbohydrates in the parenchyma cells and the role of phloem in the delivery of sugars to sustain refilling. Both enzymatic analysis and visualization techniques have demonstrated that starch levels in living parenchyma cells adjacent to xylem vessels decrease on a timescale coincident with embolism refilling (Bucci *et al*., 2003, Nardini *et al*., 2011a, Sakr *et al*., 2003, Salleo *et al*., 2009, Secchi & Zwieniecki, 2011). Furthermore, a drop in starch content has been associated with an increase in parenchyma cell sucrose content (Nardini *et al*., 2011a, Regier *et al*., 2009, Salleo *et al*., 2009, Secchi & Zwieniecki, 2010). These results are strongly supported by a transcriptome analysis that found, in response to embolism, both the down-regulation of genes transcribing the monosaccharide metabolic pathways and the strong up-regulation of those involved in the disaccharide metabolic pathways that include starch metabolism (Secchi and Zwieniecki, 2011). Similar changes in transcript expression were found in the petioles of grapevine during cycles of water stress and recovery (Perrone *et al*., 2012b). Increased rates of starch depolymerization lead to an upsurge of simple, low-molecular weight sugar concentration that may be exported across membranes into the conduit wall establishing the gradient required to drive water movement into the embolized vessel (Brodersen & McElrone, 2013, Nardini *et al*., 2011b, Secchi & Zwieniecki, 2012).

However, it has yet to be shown how the products of starch hydrolysis move from starch storing living cells in the stem to vessel-associated parenchyma and finally to the walls of the conduit network. It has been recently proposed that this process of sugar movement might be controlled by tissue-level changes in stem chemistry (Fig. 1):

- An increase in cellular sucrose concentration can be generated by either the long-distance transport of sugars (mostly sucrose) through the phloem or from the hydrolysis of starch stored in the stem; hypothesis confirmed by Bucci *et al*., (2003), Nardini *et al*., (2011a), Salleo *et al*., (2004),

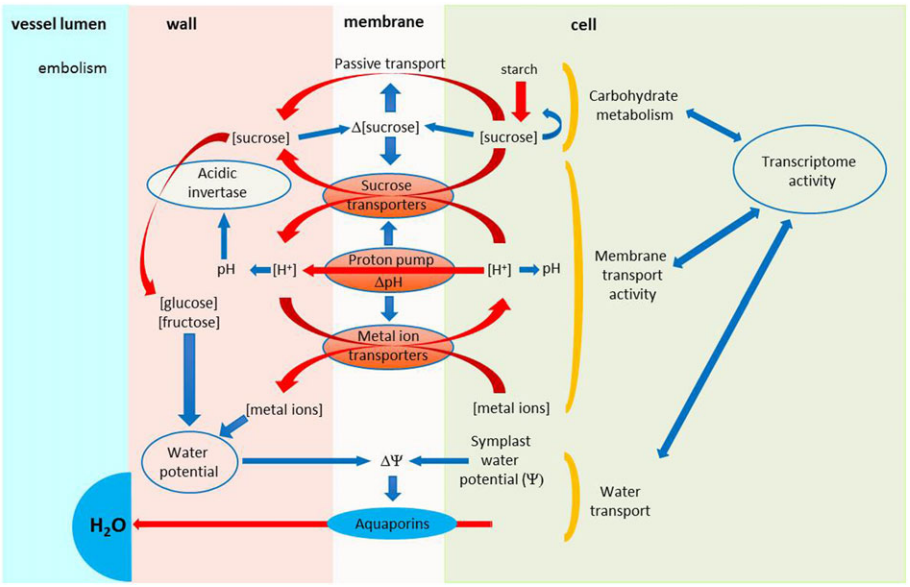

Figure 1. Schematic illustration of membrane transporter activity during onset of water stress and recovery (Secchi & Zwieniecki 2016). For details of overall scenario describing stem parenchyma cell activity, please refer to the text.

Salleo *et al.*, (2009), Secchi & Zwieniecki, (2011), and Zwieniecki & Holbrook, (2009).

- Starch hydrolysis results in an increased symplastic cellular soluble sugar concentration providing not only osmotic protection from stress but also shifting the membrane sucrose gradient. This shift may trigger proton-coupled sucrose efflux into apoplastic compartments through the plasma membrane sugar/proton co-transporters, as energized by membrane H^+-ATPase (Geiger, 2011, Wippel *et al.*, 2010); this hypothesis has to be proved directly in plants, although an active proton-sucrose efflux has been demonstrated in heterologous systems (Carpaneto *et al.*, 2005, Carpaneto *et al.*, 2010).
- Proton pumps have been localized in xylem-associated cells (De Boer & Volkov, 2003), and the chemical inhibition of H^+-ATPase pumps in parenchyma cells prevent recovery, while the stimulation of their activities induces recovery (Salleo *et al.*, 2004, Secchi & Zwieniecki, 2016), confirming proton-coupled membrane transport.
- Sucrose-proton efflux may have two effects: an increase in apoplastic sucrose concentration and a drop in apoplastic pH values. This hypothesis is supported by a previous report showing that xylem sap collected from embolized vessels in poplar had a significantly lower pH than functional conduits (Secchi & Zwieniecki, 2012). Alteration in pH is one of the first chemical changes measurable in xylem sap from plants exposed to drought (Bahrun *et al.*, 2002, Sobeih *et al.*, 2004). Although the alkalization of sap is often measured in transpiring plants (Wilkinson & Davies, 1997) and leads to the general conclusion that pH in xylem sap increases with water stress, a recent study showed that alkalization is not a universal phenomenon. It has been demonstrated that in woody plants, xylem sap alkalization is much less common than in annual species, and of the 22 species studied and exposed to water stress, only four showed an increase in sap pH (Sharp & Davies, 2009). Indeed, a new study, which uses a novel in vivo technique, has just confirmed that during embolism formation (induced water stress) xylem pH decreases (Secchi & Zwieniecki, 2016)
- A drop in pH and thus the acidification of the apoplast may stimulate the apoplastic activity of acidic invertases, promoting the reduction of sucrose concentrations and at the same time increasing the accumulation of monosaccharides (glucose and fructose; (Secchi & Zwieniecki, 2016) providing additional osmoticum for refilling.
- Lower apoplastic pH may trigger not only the activation of proton pumps but also the activation of metal ion antiporters. Xylem sap collected from embolized vessels in poplar contained up to five times more the osmotic potential of functional vessels. Inorganic ions accounted for half of the osmoticum, and the rest was derived from soluble sugars (Secchi & Zwieniecki, 2012). Evidence of metal ion contributions to osmoticum is also provided by a previous study of transcriptome analysis showing an increase in the expression level of metal ion transporters in response to embolism formation (Secchi *et al.*, 2011). Thus, sugars and ions together may account for the driving force that draws water into empty conduits under low water stress [i.e. during recovery from stress; (Secchi & Zwieniecki, 2012)]. Previously, CryoSEM showed concentration of solutes in the xylem sap of maize in functional vessels in the range of ~100 mM (McCully *et al.*, 2000; CryoSEM does not detect sugar) that is osmotic levels similar to those observed in trees and adequately high ~0.2 MPa to drive water into conduit under low tension even if no sugar was present. It is worth to mention that majority of herbaceous species undergo an alkalization of apoplastic pH during stress conditions (Sharp & Davies, 2009); therefore, sugar accumulation in the apoplast would be limited and other mechanisms of recovery from embolism might be in play. Thus, trees and crops might adopt different strategies in order to recovery from embolism

formation where trees depend on local recovery while herbaceous plants on root pressure.
- Mechanisms involved in embolism repair require that water enters empty conduits and fills the entire lumen. Indeed, visual evidence from cryo-SEM studies, MRI observations and CT-scans show that water reappears in previously empty conduits, confirming that plants do have the ability to remove embolisms in the xylem (Clearwater & Goldstein, 2005, Holbrook *et al.*, 2001, Scheenen *et al.*, 2007, Zwieniecki *et al.*, 2013). Recently, *in vivo* imaging of grapevine with high resolution x-ray computed tomography has shown that water droplets form on vessel walls in the proximity of parenchyma cells and that these droplets expand until the lumen completely refills (Knipfer *et al.*, 2015, Knipfer *et al.*, 2016, Brodersen *et al.*, 2010). This evidence supports the prediction that parenchyma cells draw water into embolized conduits.

In the proposed scenario, xylem parenchyma cells are the primary means for restoration of hydraulic continuity in the xylem. Although not previously tested, the amount of parenchyma cells, their relative contact with vessels and their physiological activity may be crucial in providing the energy for refilling. Preliminary support for this hypothesis has been provided by Choat *et al.* (2015); the authors suggested that the lack of refilling in a conifer species (*Sequoia sempervirens*) could be attributed to the lower amount of parenchyma cells. However, new evidence suggests presence of refilling activity in the same species exposed to fog (Earles *et al.*, 2016). In this case, radial parenchyma cells can provide path for water transport from the bark surface to tracheids. In addition, observations that water droplets form on vessel walls in contact with axial/radial parenchyma cells suggest that these cells may be highly active in water transport. If so, we can assume that water channel proteins (aquaporins) are critical to the refilling process, and a closer look at aquaporin physiology is required to understand the potential for refilling in trees.

AQUAPORINS AND THEIR LOCALIZATION IN THE STEM OF WOODY SPECIES

In all developmental phases and responses to environmental cues, the maintenance of water flow across membranes is regulated by the activity and abundance of aquaporins (Hachez *et al.*, 2006). Five families of AQPs are known in higher plants on the basis of sequence similarities and common association with peculiar cell membrane localization (Maurel *et al.*, 2015). Among these, the PIP family, which is in turn divided into two subfamilies, PIP1 and PIP2, is the most prolific; examples can be found in woody plants, such as grapevine and poplar, where 28 and 56 MIP-encoding genes have been identified, respectively (Fouquet *et al.*, 2008, Gupta & Sankararamakrishnan, 2009, Shelden *et al.*, 2009). In the last few years, MIP aquaporins were found to act as multifunctional pores; highlighting that AQPs are able to perceive a wide array of signals crucial to cell metabolism, nutrition and signalling cascades [for details see recent reviews by Bienert & Chaumont (2014), Kaldenhoff *et al.*, (2014), Maurel *et al.*, (2015)]. Beyond single aquaporin type functions, further efforts have also recently improved our understanding of specific interactions among AQP isoforms, such as PIP1 and PIP2 members, especially in woody species. In this context, the quantification of expression changes in response to the imposed stress of a single and/or multiple groups of AQP isoforms coupled with studies on their tissue-specific localization is certainly a relevant strategy for obtaining experimental evidence about the physiological roles of aquaporins. Indeed, according to Heinen *et al.*, (2009) there are three main ways by which AQPs regulate water movement across cell membranes: expression level, trafficking and gating. While all three are important, expression analysis in particular is being used to guide our understanding of the specific localization and activity of diverse AQP isoforms. Despite their importance in all plant species (annual and perennial) and all tissues, the majority of investigations into AQP gene functions have been carried out on herbaceous angiosperm species with special focus on leaves and roots [for details see the recent review by Maurel *et al.*, (2015)], considering the rest of a plant bulk tissue (i.e. total xylem and bark). Studies of aquaporins in woody angiosperms and the localization of AQPs in ferns and gymnosperms are much less explored. A current and comprehensive list of PIP1 and PIP2 isoforms expressed in woody plant tissues is reported in Table 1. Several isoforms are tissue-specific, and some are almost exclusively expressed in the xylem. However, phylogenetic analysis conducted on aquaporin sequences described in Table 1 show no obvious clustering (Fig. 2). The lack of a phylogenetic signal most likely precludes a simple computational approach to detect the AQPs responsible for the maintenance of xylem hydraulic capacity.

The first indications of AQP presence in the stem of woody perennials was derived from gene expression studies conducted on poplar species. Among trees, poplar is certainly the best candidate for a woody model system for molecular biology experiments addressing the functional characterization of genes such as aquaporins. Indeed, the genome of the species *Populus trichocarpa* has already been fully sequenced and released (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.htm). In addition, these plants are relatively easy to genetically transform. Despite the overall limited information on AQP activity in the stem, the majority of the available data have been obtained on poplar. Although limited in scope, analyses have shown highly variable expression in different stem sections, at different developmental stages and in response to stress treatments, thus suggesting that stem AQPs are an important part of stem biology in woody plants.

For instance, significant insights on the expression profiles of AQP isoforms were provided in poplar by Secchi *et al.*, (2009). Along with transcripts of PIP1 and PIP2 members found in roots and leaves, they have also been found in bark and xylem samples of poplar stems, although with highly diverse mixes of isoform characteristics for each tissue. Detailed investigations into expression patterns of the previously characterized *PIP1* and *PIP2* genes in *P. trichocarpa* plants responding to water stress and embolization events supports the idea that specific xylem parenchyma AQPs are induced by stress and suggests some functional role of these proteins in dealing with drought,

Table 1. List of known PIP aquaporins expressed in woody plants with focus on stem aquaporins

Gene name	Species	Gene ID NCBI GenBank[(a)]/ Phytozome[(b)]	Expression patterns	Response to embolism/ recovery	References
PtPIP1.1	*Populus trichocarpa*	POPTR_0008s06580 (b)	L (+++), R (++), W (++), B (+++)	**	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP1.2	*P. trichocarpa*	POPTR_0003s12870 (b)	L (++), R (++), W (++), B (++)	no changes	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP1.3	*P. trichocarpa*	POPTR_0010s19930 (b)	L (+++), R (++), W (++)B (+++)	*	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP1.4	*P. trichocarpa*	POPTR_0006s09920 (b)	L (+), R (+), W (+), B (+)	*	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP1.5	*P. trichocarpa*	POPTR_0016s12070 (b)	L (++), R (++), W (++)B (++)	*	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP2.1	*P. trichocarpa*	POPTR_0009s13890 (b)	L (+), R (+), W (+), B (+)	*	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP2.2	*P. trichocarpa*	POPTR_0004s18240 (b)	L (+++), R (+++), W (+++), B (+++)	*	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP2.3	*P. trichocarpa*	POPTR_0016s09090 (b)	L (+), R (+), W (++), B (++)	**	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP2.4	*P. trichocarpa*	POPTR_0010s22950 (b)	L (++), R (++), W (++), B (++)	*	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP2.5	*P. trichocarpa*	POPTR_0006s12980 (b)	L (+++), R (+++), W (++), B (+)	*	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP2.6	*P. trichocarpa*	POPTR_0008s03950 (b)	L (+++), R (++), W (++), B (++)	**	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP2.7	*P. trichocarpa*	POPTR_0009s01940 (b)	L (++), R (+++), W (+), B (++)	*	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
PtPIP2.8	*P. trichocarpa*	POPTR_0005s11110 (b)	L (+++), R (+), W (+), B (++)	**	(Secchi *et al.*, 2009, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011)
OePIP2.1	*Olea europea L*	DQ202709 (a)	L (+), R (+++), T (+)	**	(Secchi *et al.*, 2007a, Secchi *et al.*, 2007b)
OePIP1.1	*O. europea L*	DQ202708 (a)	L (+), R (++), T (++)	**	(Secchi *et al.*, 2007a, Secchi *et al.*, 2007b)
PttPIP2.1	*P. tremula · tremuloides*	AJ849324 (a)	L (++), S (++), MR (++), FR (++)		(Marjanovic *et al.*, 2005)
PttPIP2.2	*P. tremula · tremuloides*	AJ849325 (a)	L (+), S (+), MR (++), FR (+++)		(Marjanovic *et al.*, 2005)
PttPIP2.3	*P. tremula · tremuloides*	AJ849326 (a)	L (+), S (+), MR (++), FR (+)		(Marjanovic *et al.*, 2005)
PttPIP2.4	*P. tremula · tremuloides*	AJ849327 (a)	L (++), S (+), MR (+), FR (++)		(Marjanovic *et al.*, 2005)
PttPIP2.5	*P. tremula · tremuloides*	AJ849328 (a)	L (+), S (−), MR (+), FR (++)		(Marjanovic *et al.*, 2005)
PttPIP1.1	*P. tremula · tremuloides*	AJ849323 (a)	L (+), S (+), MR (++), FR (+++)		(Marjanovic *et al.*, 2005)
PttPIP1.2	*P. tremula · tremuloides*	AJ849322 (a)	L (+), S (++), MR (++), FR (++)		(Marjanovic *et al.*, 2005)
JrPIP2.1	*Juglans regia*	AY189974 (a)	L (+++), B (++), W (++), R (+)	**	(Sakr *et al.*, 2003)
JrPIP2.2	*J. regia*	AAO39008 (a)	L (+++), B (+), W (+), R (+++)	**	(Sakr *et al.*, 2003)
PtPIP2.3	*Populus* × 'Okanese'	567607 (b)	P (++), C (+++), R(CC) (+++), R(IC) (+)	**	(Almeida-Rodriguez & Hacke, 2012)
PtPIP2.5	*Populus* × 'Okanese'	826419 (b)	P (++), C (+++), R(CC) (+++), R(IC) (+)	**	(Almeida-Rodriguez & Hacke, 2012)
PgPIP1;1	*Picea glauca*	BT113218 (a)	R, L, S, W	*	(Laur & Hacke, 2014)
PgPIP1;2	*P. glauca*	BT115139 (a)	R, L, S	**	(Laur & Hacke, 2014)
PgPIP1;3	*P. glauca*	BT105794 (a)	S, L		(Laur & Hacke, 2014)
PgPIP2;1	*P. glauca*	BT107672 (a)	R, L, S	*	(Laur & Hacke, 2014)
PgPIP2;2	*P. glauca*	BT105999 (a)	R, L, S, W	**	(Laur & Hacke, 2014)
PgPIP2;3	*P. glauca*	BT115639 (a)	R, S		(Laur & Hacke, 2014)
PgPIP2;5	*P. glauca*	BT108646 (a)	reproductive parts		(Laur & Hacke, 2014)

(*Continues*)

Table 1. (Continued)

Gene name	Species	Gene ID NCBI GenBank[(a)]/ Phytozome[(b)]	Expression patterns	Response to embolism/ recovery	References
PgPIP2;7	*P. glauca*	BT106222 (a)	R, S		(Laur & Hacke, 2014)
PgPIP2;8	*P. glauca*	BT106086 (a) (partial sequence)	R, L, S		(Laur & Hacke, 2014)
PgPIP2;9	*P. glauca*	BT106471 (a)	S		(Laur & Hacke, 2014)
PgPIP2;10	*P. glauca*	BT106822 (a)	R, S		(Laur & Hacke, 2014)
PgPIP2;11	*P. glauca*	BT106775 (a)	S		(Laur & Hacke, 2014)
PgPIP2;12	*P. glauca*	BT106446 (a)	R, S		(Laur & Hacke, 2014)
PgPIP2;13	*P. glauca*	BT110135 (a)	S		(Laur & Hacke, 2014)
CaPIP2;1	*Coffea arabica*	LM654169 (a)	L, R	**	(Miniussi *et al*., 2015)
CaPIP2;2	*C. arabica*	LM654170 (a)	L, R	**	(Miniussi *et al*., 2015)
CaPIP1;1	*C. arabica*	LM654171 (a)	L, R	**	(Miniussi *et al*., 2015)
CaPIP1;2	*C. arabica*	LM654172 (a)	L, R	**	(Miniussi *et al*., 2015)
ThPIP1-2	*Tamarix ramosissima*	Unigene23675 (a)	L	*	(Yan *et al*., 2015)
ThPIP2-1	*T. ramosissima*	Unigene38680 (a)	L	*	(Yan *et al*., 2015)
QpPIP2:1	*Quercus petraea*, *Quercus robur*	JQ768372 (a)	R (+++)		(Rasheed-Rasheed-Depardieu *et al*., 2012)
QpPIP2:2	*Q. petraea*, *Q. robur*	JQ846268 (a)	R (++)		(Rasheed-Depardieu *et al*., 2012)
QpPIP2:3	*Q. petraea*, *Q. robur*	JQ846269 (a)	R (+)		(Rasheed-Depardieu *et al*., 2012)
QpPIP1:1	*Q. petraea*, *Q. robur*	JQ846270 (a)	R (++)		(Rasheed-Depardieu *et al*., 2012)
QpPIP1:2	*Q. petraea*, *Q. robur*	JQ846271 (a)	R (++)		(Rasheed-Depardieu *et al*., 2012)
QpPIP1:3	*Q. petraea*, *Q. robur*	JQ846272 (a)	R (−)		(Rasheed-Depardieu *et al*., 2012)
MusaPIP1;2	*Musa acuminata*	FF561783 (a)	L (++)		(Sreedharan *et al*., 2013)
MusaPIP2;6	*M. acuminata*	FL667907 (a)	R, L		(Sreedharan *et al*., 2015)
EcPIP1	*Eucalyptus grandis*	XM_010057362.1 (a)	L (+)		(Tsuchihira *et al*., 2010)
EcPIP2	*Eucalyptus camaldulensis*	XM_010033696.1 (a)	L (+)		(Tsuchihira *et al*., 2010)
CsPIP1;1	*Citrus sinensis*	orange1.1 g018895 (b)	R (+), L (+++), FL (+++), CL (+++), F (+++)		(de Paula Santos Martins *et al*., 2015)
CsPIP1;2	*C. sinensis*	orange1.1 g023021 (b)	R (+), L (+), FL (++), CL (++), F (++)		(de Paula Santos Martins *et al*., 2015)
CsPIP1;3	*C. sinensis*	orange1.1 g023107 (b)	R (+), L (+++), FL (+++), CL (++), F (++)		(de Paula Santos Martins *et al*., 2015)
CsPIP1;4	*C. sinensis*	orange1.1 g023069 (b)	R (+), L (++), FL (+++), CL (−), F (++)		(de Paula Santos Martins *et al*., 2015)
CsPIP2;1	*C. sinensis*	orange1.1 g023108 (b)	R (+), L (+), FL(−), CL (−), F (+)		(de Paula Santos Martins *et al*., 2015)
CsPIP2;2	*C. sinensis*	orange1.1 g022966 (b)	R (+), L (++), FL (++), CL (++), F (++)		(de Paula Santos Martins *et al*., 2015)
CsPIP2;3	*C. sinensis*	orange1.1 g019681 (b)	R (+), L (+++), FL (+++), CL (++), F (+++)		(de Paula Santos Martins *et al*., 2015)
CsPIP2;4	*C. sinensis*	orange1.1 g023370 (b)	R (+), L (+++), FL (+++), CL (+), F (+)		(de Paula Santos Martins *et al*., 2015)
PpPIP1	*Prunus persica*	AB303644 (a)	Bu	Cold stress	(Yooyongwech *et al*., 2009)
PpPIP2	*P. persica*	AB329725 (a) (partial seq)	Bu	Cold stress	(Yooyongwech *et al*., 2009)
VvPIP1;1	*Vitis hybrid Richter-110*	AF141643 (a)	R (+++), RT (+++), L (+), S (+)		(Baiges *et al*., 2001, Galmes *et al*., 2007)
VvPIP1;2	*V. hybrid Richter-110*	AF141898 (a)	R (+), RT (+), L (+), S (++)		(Baiges *et al*., 2001, Galmes *et al*., 2007)
VvPIP1;3	*V. hybrid Richter-110*	AF141899 (a)	R (++), RT (++), L (+), S (++)		(Baiges *et al*., 2001, Galmes *et al*., 2007)
VvPIP2;1	*V. hybrid Richter-110*	AF141642 (a)	R (++), RT (+++), L (++), S (++)		(Baiges *et al*., 2001, Galmes *et al*., 2007)

(*Continues*)

Table 1. (Continued)

Gene name	Species	Gene ID NCBI GenBank[(a)]/ Phytozome[(b)]	Expression patterns	Response to embolism/ recovery	References
VvPIP2;2	*V. hybrid Richter-110*	AF141900 (a)	R (++), RT (+++), L (++), S (+++)		(Baiges *et al*., 2001, Galmes *et al*., 2007)
VvPIP1a	*Vitis vinifera* cv. Chardonnay, Ugny Blanc, Pinot Meunier	AF188844 (a)	F (++)		(Picaud *et al*., 2003)
VvPIP1b	*V. vinifera* cv. Chardonnay, Ugny Blanc, Pinot Meunier	AF188843 (a)	F (++)		(Picaud *et al*., 2003)
VvPIP1;1	*V. vinifera* cv. Cabernet Sauvignon	DQ834694 (a)	F (+)		(Fouquet *et al*., 2008)
VvPIP1;2	*V. vinifera* cv. Cabernet Sauvignon	DQ834695 (a)	F (+)		(Fouquet *et al*., 2008)
VvPIP1;3	*V. vinifera* cv. Cabernet Sauvignon	DQ834696 (a)	F (+++)		(Fouquet *et al*., 2008)
VvPIP2;1	*V. vinifera* cv. Cabernet Sauvignon	DQ834698 (a)	F (+++)		(Fouquet *et al*., 2008)
VvPIP2;2	*V. vinifera* cv. Cabernet Sauvignon	DQ834699 (a)	F (+)		(Fouquet *et al*., 2008)
VvPIP2;3	*V. vinifera* cv. Cabernet Sauvignon	DQ834700 (a)	F (++)		(Fouquet *et al*., 2008)
VvPIP1;1	*V, vinifera* diverse cvs	EF364432 (a)	R (−, cv Nebbiolo; ++ cv. Grenache and Chardonnay), L (+), S (−), P (++)	**	(Chitarra *et al*., 2014, Perrone *et al*., 2012a, Perrone *et al*., 2012b, Pou *et al*., 2013, Shelden *et al*., 2009, Vandeleur *et al*., 2009)
VvPIP1;2	*V. vinifera* diverse cvs	EF364433 (a)	R (+), S (++), P(−)	*	(Vandeleur *et al*., 2009, Perrone *et al*., 2012b, Chitarra *et al*., 2014, Shelden *et al*., 2009)
VvPIP1;4	*V. vinifera* diverse cvs	EF364435 (a)	R (++)		(Vandeleur *et al*., 2009, Shelden *et al*., 2009)
VvPIP2;1	*V. vinifera* diverse cvs	AY823263 (a)	R (+), L (++), S (++), P (++)	**	(Vandeleur *et al*., 2009, Perrone *et al*., 2012a, Perrone *et al*., 2012b, Chitarra *et al*., 2014, Pou *et al*., 2013, Shelden *et al*., 2009)
VvPIP2;2	*V. vinifera* diverse cvs	EF364436 (a)	R (++), L (++), S (++)	**	(Vandeleur *et al*., 2009, Perrone *et al*., 2012a, Perrone *et al*., 2012b, Pou *et al*., 2013, Shelden *et al*., 2009)
VvPIP2;3	*V. vinifera* diverse cvs	EF364437 (a)	R (+), L (−, cv. Nebbiolo; ++, cv. Chardonnay)	**	(Vandeleur *et al*., 2009, Perrone et *al*., 2012a, Pou *et al*., 2013, Shelden *et al*., 2009)
VvPIP2;4	*V. vinifera* diverse cvs	EF364438 (a)	R (+)		(Vandeleur *et al*., 2009, Shelden *et al*., 2009)
VvPIP2;4 N	*V. vinifera* diverse cvs	DQ358107 (a)	R (+++), L (+), S (−), P (++)	**	(Perrone *et al*., 2012a, Perrone *et al*., 2012b, Chitarra *et al*., 2014)

L, leaf; R, root; MP, main root; FR, fine root; RT, root tip; W, wood/xylem; B, bark; S, stem (wood and bark); T, twig; P, phloem (mainly in companion cells); C, cambial zone and derivatives; R, ray parenchyma; R(CC), ray contact cells; R(IC), ray, isolation cells; Bu, buds; FL, flower; F, Fruit; CL, Callus

+++, strong expression; ++, expression; +, detectable expression; −, no expression

*not significant changes in gene expression

**significant changes in gene expression

embolism formation and recovery (Secchi *et al*., 2011, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011). In particular, an analysis of the temporal dynamics of expression of all *PIP1* and *PIP2* transcriptional profiles, found a general strong over-expression of the PIP1 subfamily when water stress occurred. However, response to embolism formation has resulted in the selective activation of only *PIP1;1* and *PIP1;3* genes in stem parenchyma (Secchi & Zwieniecki, 2010). Genome-wide analysis of *P. trichocarpa* responding to embolism formation not only confirm the specificity of AQP expression patterns but also show a correlation with changes in the expression of genes tied to carbohydrate metabolism and sugar transport (Secchi *et al*., 2011).

Similarly, in a work performed on grapevine plants (*Vitis vinifera* cv Grenache) subjected to either drought stress or artificially induced embolization, changes in the expression of diverse PIP1 and PIP2 aquaporin genes were profiled in both petioles (whole tissue level) and also in vessel associated cells (VACs) isolated from the same tissue samples using a laser micro-dissection technique (Chitarra *et al*., 2014). Here, the comparison of AQPs expression trends in a target cell type coupled with the transcriptional profiles of the same genes at

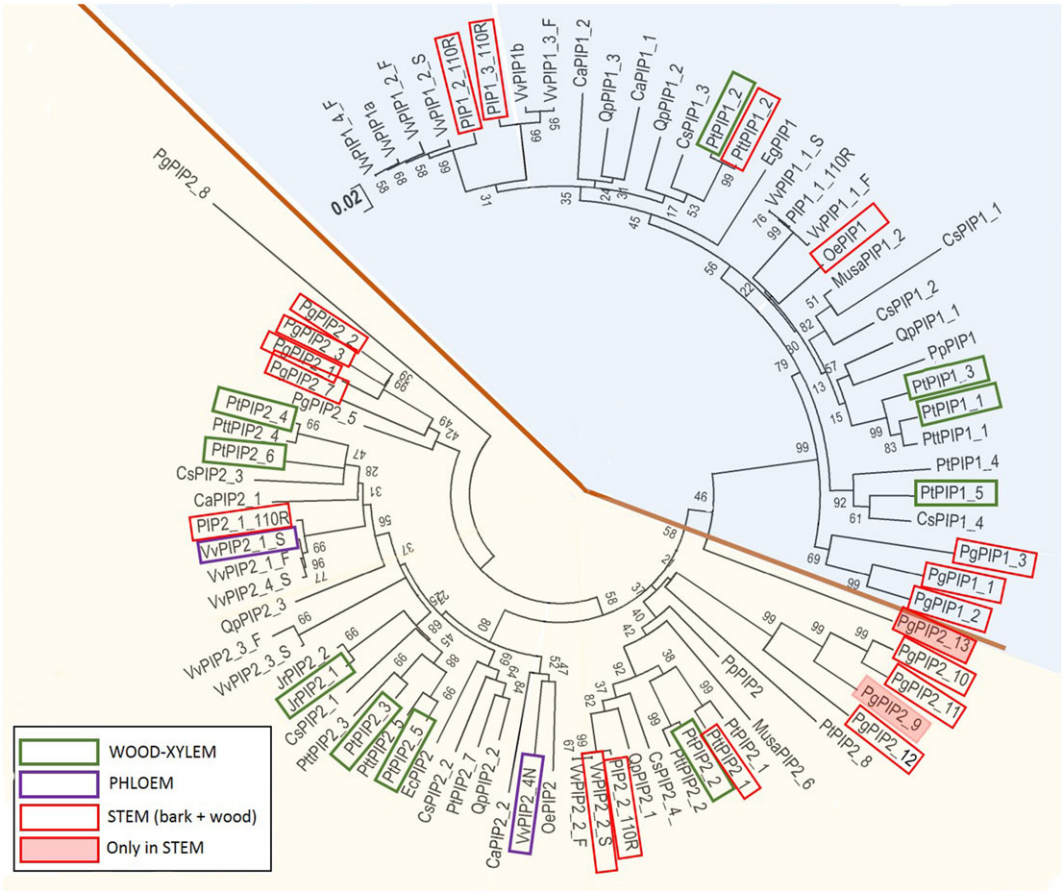

Figure 2. Neighbour-joining circle tree of the woody plant PIP1-type and PIP2-type aquaporin proteins detailed in Table 1. Only for *Vitis* aquaporins, the letters 110R, F or S at the end of the protein name identify the aquaporins characterized by Baiges *et al.* (2001), Fouquet *et al.* (2008) and Shelden *et al.* (2009), respectively. The significance of each node was tested using 2000 bootstrap replicates. Accession numbers of each aquaporin are reported in Table 1 together with expression details and references. The green rectangular indicate, respectively, the specific PIP expression (++ or +++) in xylem, purple in the phloem and red in entire stem tissue (bark and wood). The filled red rectangulars denote the presence of PIP expression exclusively in stem tissue (it was not detectable in the other tissues tested).

the whole tissue level provided the first consistent information about the specificity of some of these transcripts. Indeed, while some of the *VvPIP1* and *VvPIP2*-analysed genes were on the whole induced by stress and subsequent recovery in entire petioles, two transcripts encoding *VvPIP1;1* and *VvPIP2;4 N*, were exclusively activated in VACs, suggesting that the activity of the two derived proteins was restricted to these cells (Chitarra *et al.*, 2014).

Although transcriptomic methods are certainly essential to the unravelling of AQP transcript alterations occurring in target organs or tissues during either plant phenological phases or the application of stress, they can only provide indirect evidence on the functional role of these genes. They do not supply information about transcript localization. To better address the characterization of aquaporins, the application of tissue and cellular level localization studies is pivotal. The first cellular detection of AQPs in the stem of trees was in walnut (*Juglans regia*) plants experiencing winter embolism formation. The expression signal of two members of the PIP2 subfamily (*JrPIP2.1* and *JrPIP2.2*) was analysed by an immunolocalization technique on several divisions of xylem tissue including vessels, fibres and parenchyma cells (Sakr *et al.*, 2003). The immunolabeling results showed that the strongest detection of the two proteins occurred in the living parenchyma cells in direct contact with xylem vessels (VACs), clearly attesting that during winter months these AQPs are specifically located in the VACs of walnut stems.

Subsequent tissue-specific and cell-specific localizations of three AQP genes (two PIP2 isoforms and one TIP) in the secondary xylem of hybrid poplar stems directly observed through *in situ* hybridization experiments (Almeida-Rodriguez & Hacke, 2012) showed an increase in the abundance of all three in parenchyma cells when drought or high N fertilization was applied. This study validated and visually confirmed a previous work based solely on gene expression data (Hacke *et al.*, 2010). Drought and N fertilization resulted in significant changes in the abundance of target AQP transcripts in living tissues of the stem as well as in the ray cells adjacent to vessels (pith parenchyma), with various degrees of changes in expression patterns depending on the applied treatment. Surprisingly, this research revealed a strong increase in the AQP signal of parenchyma cells (often isolated) but only upon drought, suggesting an increased potential for water exchange between apoplast and symplast in response to imposed external conditions.

Finally, AQP tissue-localization studies in non-angiosperms (gymnosperms and ferns) are almost exclusively focused on leaves and gametophytes. For instance, interesting data were reported by Laur & Hacke (2014) in a study dealing with the analysis of aquaporin expression in the needles of *Picea glauca*, where the expression of PIP1 and PIP2 aquaporins was measured and compared with immunolocalization and *in situ* hybridization experiments. The authors indicated that upon water deficit, all tested PIP genes were significantly down-regulated in needles while a high humidity treatment resulted in an increased expression level for all transcripts, but to different extents depending on the period of exposure. These results were confirmed by the diverse patterns of tissue localization obtained for each AQP. Indeed, while PIP1s were mainly localized in the endodermis, PIP2s showed a diffused signal within the central cylinder of the needle in both phloem tissue and in transfusion parenchyma cells. It was suggested that PIP1 isoforms mainly regulate water transport across the bundle sheath, from the needle epidermis towards the vascular tissue, while PIP2s may facilitate water movement from the needle towards stems (Laur & Hacke, 2014). These findings are consistent with previous aquaporin immunolocalization experiments carried out in *P. abies*, where a strong signal was detected in needles at the vascular tissue level (Oliviusson *et al.*, 2001) and by observations of a higher abundance of PIP1 and PIP2 aquaporins at the endodermis and phloem cell level in the needles of *P. abies* recovering from winter embolism (Mayr *et al.*, 2014). Information on AQP expression in ferns comes from work conducted on the xerophytic fern *Cheilanthes lanosa* (Diamond *et al.*, 2012) where analysis indicated that the role of PIP1 proteins is highlighted by the maintenance of water balance during gametophyte stages.

Despite limited information, we can conclude that AQPs are abundant in living cells associated with long distance transport tissue, including xylem axial and radial parenchyma and phloem. It is also apparent that the expression of stem AQPs is related to plant hydraulic status with drought causing species/tissue specific up or down regulation and recovery from stress (rain, re-watering and fog) causing significant up-regulation of stem specific AQPs. This pattern of expression underlines the potential role of AQPs in the recovery of the hydraulic capacity of the xylem, a trait that long-lived perennial plants may rely upon for their survival.

PARENCHYMA AQUAPORINS AND RECOVERY FROM WATER STRESS

Existing models of recovery processes occurring in trees indicate that, among other functions, living parenchyma cells associated with xylem conduits are key players in both supplying the water and generating the energy needed to refill non-functional vessels (Brodersen & McElrone, 2013, Nardini *et al.*, 2011b, Salleo *et al.*, 2004a, Zwieniecki & Holbrook, 2009). As water flow between the symplast and apoplast is mediated by aquaporins, xylem parenchyma cells possess a significant ability to temporally and spatially control water efflux, by regulating the expression and activity of specific AQP isoforms. As mentioned previously, AQP *in vivo* localization studies have revealed a fine-tuning of AQP expression in vessels associated cells (VACs) especially during transition periods between drought stress and stress recovery. It is thus believed that the physiological function of AQPs is specifically needed not during the imposition of environmental stress (drought and frost), but during the recovery from stresses that often requires the restoration of xylem hydraulic conductivity.

The contribution of aquaporins to the restoration of xylem hydraulic conductivity throughout periods of water stress and/or subsequent recovery have mainly been addressed in order to better understand the plant water relations of distal organs (roots and leaves) (Perrone *et al.*, 2012a, Perrone *et al.*, 2012b, Pou *et al.*, 2013, Tsuchihira *et al.*, 2010), whereas a comprehensive understanding of AQPs in controlling xylem refilling in the stem is just emerging. Revisiting the insights gained from walnut stems in which expression was correlated with embolism recovery processes (Sakr *et al.*, 2003), both over-expression and an increased abundance of two walnut PIP2 proteins was exclusively induced in winter months in the VACs. This increase was simultaneous with an increase in the sucrose concentrations of xylem sap and a decrease of starch content in parenchyma cells (Sakr *et al.*, 2003). It was assumed that over-expression and increases in AQP abundance facilitate water flow from VACs into embolized vessels following this newly generated osmotic gradient. These results support earlier observations of increased xylem water content, which in parallel decreases in stem parenchyma water content reflecting the flow of water from cells to embolized vessels (Ameglio *et al.*, 2001). Recently, additional reports have highlighted the involvement of PIP2 genes in particular in facilitating the recovery process (Table 1). For instance, the increased abundance of *PIP2;3* and *PIP2;5* detected in the VACs of drought-exposed poplar stems (Almeida-Rodriguez & Hacke, 2012) and the over-expression of *PIP2;4 N* and *PIP2;1* genes observed in the VACs of either embolized or recovering grape petioles (Chitarra *et al.*, 2014) may both support the need for PIP2 activity during vessel refilling along the xylem-VAC-phloem transport path. These observations are in agreement with the current models of embolism repair involving the interaction of xylem and phloem cells (Nardini *et al.*, 2011a, Secchi *et al.*, 2011, Secchi & Zwieniecki, 2016) presented here (Fig. 1).

The contribution of PIP1s to water stress and recovery in trees was initially less considered as, unlike PIP2s, PIP1s were thought to have little to no water transport activity when individually expressed in *Xenopus* oocytes (Chrispeels *et al.*, 2001). This implied that PIP1 proteins did not work as water channels, and it was consequently assumed that they were not necessary for promoting transmembrane water flow. Nevertheless, it was later shown that membrane water permeability significantly increased when PIP1s were co-expressed with PIP2s, implying a physical interaction between the two (Fetter *et al.*, 2004, Secchi *et al.*, 2009, Zelazny *et al.*, 2007). Interestingly, only PIP1 genes were shown to undergo strong transcriptional increase upon water stress and embolism formation in poplar stems (Secchi *et al.*, 2011, Secchi & Zwieniecki, 2010, Secchi & Zwieniecki, 2011). In addition, the transcripts encoding *PIP1.1* and *PIP1.3* were the most expressed among PIP

aquaporin genes in poplar stems (Secchi *et al.*, 2009). These two genes were highly up-regulated in response to artificially-induced embolism, and their expression was repressed when plants recovered from embolism, showing VACs ability to sense both embolism formation and end of the refilling process (Secchi & Zwieniecki, 2010). Similarly, a *Vitis PIP1*;*1* gene was reported to be activated in VACs during both embolism formation and recovery, whereas the same transcript was not detected at the whole tissue level (Chitarra *et al.*, 2014, Perrone *et al.*, 2012b, see also Table 1). These findings demonstrate that PIP1 expression is affected by drought stress, embolism and duration of recovery from stress. However, hypotheses about PIP1s role in refilling remain open as all evidence is based on transcription analyses, and no direct proof exists on the physiological activity of these proteins.

Reverse genetic techniques have successfully been applied to the functional characterization of AQP genes mainly in herbaceous species (Aharon *et al.*, 2003, Da Ines *et al.*, 2010, Kaldenhoff *et al.*, 1998, Martre *et al.*, 2002, Postaire *et al.*, 2010) and more recently in woody plants (Bi *et al.*, 2015, Perrone *et al.*, 2012a, Secchi & Zwieniecki, 2013, Sreedharan *et al.*, 2013, Tsuchihira *et al.*, 2010). A similar reverse genetic approach was used to generate transgenic poplar lines by down-regulating the whole PIP1 gene family, with the goal of attesting the function of this PIP group by gaining *in vivo* evidence of their role during xylem embolism and recovery (Secchi & Zwieniecki, 2014). The down-regulation of PIP1s did not affect plant behaviour under well-watered conditions (Secchi & Zwieniecki, 2013), but it changed the physiological response of poplar during the progression of water stress. Specifically, the suppression of PIP1 expression activity significantly lowered refilling activity, resulting in an apparent increase in the vulnerability to embolism formation of transgenic plants (Secchi & Zwieniecki, 2014). These findings have clearly elucidated that, under water stress, the function of stem PIP1s is pivotal to both the maintenance of xylem transport capacity under stress and plant recovery from stress.

In conifers, the occurrence of freeze-thaw induced embolism formation and recovery processes is also documented (Limm *et al.*, 2009, Mayr *et al.*, 2006, Mayr *et al.*, 2002, Sparks & Black, 2000, Sparks *et al.*, 2001), but to date, very few research reports have provided experimental evidence that these phenomena can affect AQP gene regulation. In *P. abies*, frost-induced embolism has been shown to significantly correlate with the accumulation of PIP1 and PIP2-type proteins in needle endodermal and phloem cells (Mayr *et al.*, 2014). In *P. glauca*, AQPs were shown to promote xylem refilling by facilitating water movement between VACs and the embolized xylem (Laur & Hacke, 2014). Although more experimental efforts are needed to deepen existing knowledge of conifer AQP roles in recovery from embolism, the findings described previously provide some indication that gymnosperms are capable of sensing and managing xylem embolism.

FUTURE DIRECTIONS

Despite research efforts, our understanding of the biophysical and cellular mechanisms responsible for embolism refilling in woody plants remains incomplete. General agreement exists that the living parenchyma cells associated with xylem conduits are involved in the recovery process from stress. They are credited with supplying water to fill the void and releasing osmotically active compounds to generate energy gradients needed to pull water from living cells and restore hydraulic function (Nardini *et al.*, 2011a, Salleo *et al.*, 2004, Zwieniecki & Holbrook, 2009). Thus, we can postulate that a correlation between a plant's capacity to recover from severe drought and VAC volume, surface area or bridges provided between phloem and xylem should also exist. If so, more detailed anatomical studies of xylem parenchyma in combination with species drought tolerance may lead to of the discovery of interesting patterns linking drought tolerance and parenchyma activity evolution. As xylem refilling process might require water transport from living cells to xylem lumens, reductions of membrane hydraulic resistance would be beneficial during recovery from stress and thus observing patterns of expression and activity of specific AQP isoforms in living parenchyma cells might provide further clues to biology of stem under drought conditions. So far, only a handful of species have been studied, and we can neither address the question of how common described expression patterns are among species, nor provide a comprehensive overview of specific PIPs involved in the recovery process.

Another important aspect of plant recovery from severe water stress that includes the restoration of xylem hydraulic capacity is related to signalling (triggers) for the biological responses of VACs. Although we can observe changes in VAC physiological and expression activity, we do not know what physical or chemical changes trigger these responses. Several ideas have been suggested like mechano-sensing of high frequency sound waves associated with embolism (Salleo *et al.*, 2008), changes in sucrose concentration in the xylem apoplast (Nardini *et al.*, 2011a, Secchi & Zwieniecki, 2011) or changes in pH (Secchi & Zwieniecki, 2016). In this review's model (Fig. 1), sucrose concentration is the proposed trigger for embolism repair processes as previous results suggest that increased sucrose concentration in the xylem follows an expression pattern similar to that of VAC gene expression in response to the formation of embolism (Secchi & Zwieniecki, 2011). A more recent work also suggests that xylem apoplastic pH may be a significant part of the signalling path responsible for refilling apart from its role in invertase activity and sugar accumulation (Secchi & Zwieniecki, 2016). Low pH was shown to affect aquaporin molecular gating (Maurel *et al.*, 2015, Tornroth-Horsefield *et al.*, 2006, Tournaire-Roux *et al.*, 2003), which depends on protonation of the conserved amino acid residue of loop D (His193 in SoPIP2;1). At acidic pH, charged hydrogen interacts with two additional amino acids (Asp28 and Glu31) and loop B (Ser115) to stabilize loop D in a closed pore conformation (Tornroth-Horsefield *et al.*, 2006). This activity has been observed in heterologous systems (*Xenopus* oocytes) or in herbaceous species (spinach and Arabidopsis) (Tornroth-Horsefield *et al.*, 2006, Tournaire-Roux *et al.*, 2003). Because xylem apoplastic pH triggers multiple parts of the recovery process (i.e. membrane sucrose transport, AQP gating and the activity of apoplastic invertase), it is crucial to study the

in vivo chemistry of xylem and VACs following a whole system approach.

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