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Invited Expert Review

The Plant Vascular System: Evolution, Development and Functions[F]

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Contents

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Abstract

The emergence of the tracheophyte-based vascular system of land plants had major impacts on the evolution of terrestrial biology, in general, through its role in facilitating the development of plants with increased stature, photosynthetic output, and ability to colonize a greatly expanded range of environmental habitats. Recently, considerable progress has been made in terms of our understanding of the developmental and physiological programs involved in the formation and function of the plant vascular system. In this review, we first examine the evolutionary events that gave rise to the tracheophytes, followed by analysis of the genetic and hormonal networks that cooperate to orchestrate vascular development in the gymnosperms and angiosperms. The two essential functions performed by the vascular system, namely the delivery of resources (water, essential mineral nutrients, sugars and amino acids) to the various plant organs and provision of mechanical support are next discussed. Here, we focus on critical questions relating to structural and physiological properties controlling the delivery of material through the xylem and phloem. Recent discoveries into the role of the vascular system as an effective long-distance communication system are next assessed in terms of the coordination of developmental, physiological and defense-related processes, at the whole-plant level. A concerted effort has been made to integrate all these new findings into a comprehensive picture of the state-of-the-art in the area of plant vascular biology. Finally, areas important for future research are highlighted in terms of their likely contribution both to basic knowledge and applications to primary industry.

Keywords: Evolution; vascular development; phloem; xylem; nutrient delivery; long-distance communication; systemic signaling.

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Introduction

The plant vascular system carries out two essential functions, namely the delivery of resources (water, essential mineral nutrients, sugars and amino acids) to the various plant organs, and provision of mechanical support. In addition, the vascular system serves as an effective long-distance communication system, with the phloem and xylem serving to input information relating to abiotic and biotic conditions above and below ground, respectively. This combination of resource supply and delivery of information, including hormones, peptide hormones, proteins and RNA, allows the vascular system to engage in the coordination of developmental and physiological processes at the whole-plant level.

Over the past decade, considerable progress has been made in terms of our understanding of the developmental and physiological programs involved in the formation and function of the plant vascular system. In this review, we have made every effort to integrate these new findings into a comprehensive picture of the state-of-the-art in this important facet of plant biology. We also highlight potential areas important for future research in terms of their likely contribution both to basic knowledge and applications to primary industry.

Evolution of the Plant Vascular System

Why the need for a vasculature system?

For plants as photosynthetic autotrophs, the evolutionary step from uni- to multi-cellularity conferred an important selective advantage in terms of division of labor; i.e., functional specialization of tissues/organs to more effectively extract, and compete for, essential resources in aquatic and terrestrial environments. Successful colonization of terrestrial environments, by plants, depended upon positioning of organs in both aerial and soil environments to meet their autotrophic requirements. For example, for photosynthetic efficiency, sufficient levels of light only co-occur with a supply of CO_2 in aerial

environments, whereas water and mineral requirements are primarily acquired from soil environments. Thus, aerial and soil organs of early land plants were nutritionally interdependent and, consequently, there was intense selection pressure for the evolution of an inter-organ transport system to allow access to the complete spectrum of essential resources for cell growth and maintenance.

An important feature of early multicellular plants was the acquisition of plasmodesmata (PD), whose cytoplasmic channels established a symplasmic continuum throughout the body of the plant (Lucas et al. 1993). This symplasm allowed for the exchange of nutrients between the different plant organs. However, this symplasmic route, in which intracellular cytoplasmic streaming is arranged in series with intercellular diffusion through PD, is effective only over rather short distances. For example, a PD-mediated sucrose flux of 2×10^{-4} mol m^{-2} s^{-1} into heterotrophic cells would satisfy their metabolic demand. Maximum reported permeability coefficients for sucrose diffusion through PD are on the order of 6×10^{-6} m s^{-1} (Fisher and Wang 1995). Using this value, diffusion theory predicts that a significant sucrose concentration drop would be required, across each adjoining cell wall interface, to sustain this flux of sucrose from the autotrophic (photosynthetic) cells into the heterotrophic (water and mineral nutrient acquiring) cells. Thus, the path length would be limited to only a few cells, arranged in series, and the size of the organism would be limited to a few millimeters.

In order for multicellular autotrophs to overcome these diffusion-imposed size constraints, a strong selection pressure existed to evolve an axially-arranged tissue system, located throughout the plant body, with a greatly increased conductivity for intercellular transport. The solution to this problem began over 470 Mya and, in combination with prevailing global climate change, including dramatic changes in atmospheric CO_2 levels, gave rise to the development of the cuticle and stomata, important adaptations that both reduced tissue dehydration and increased the capacity for exchange of CO_2, thereby enhancing the rates of photosynthesis (Franks and Brodribb 2005; Ruszala et al. 2011; but see Duckett et al. 2009). Following acquisition of these two traits, early land plants evolved cells specialized for long-distance transport of food and water (Ligrone et al. 2000, 2012; Raven 2003; van Bel 2003; Pittermann 2010). Irrespective of plant group, these cells became arranged end-to-end in longitudinal files having a simplified cytoplasm and modified end walls designed to increase their intra- and intercellular conductivities, respectively.

In land plants, the degree of cellular modifications of transport cells increases from the bryophytes (pretracheophytes—also termed non-vascular plants—the liverworts, mosses and hornworts), to the early tracheophytes, the vascular cryptogams (lycophytes and pterophytes), on through to seed plants (Ligrone et al. 2000, 2012; Raven 2003; van Bel 2003). These cell specializations neatly scale with maximal sizes attained by each group of land plants. Interestingly, impacts of enhancing conductivities of cells transporting sugars converges with a greater influence imposed by evolving water conducting cells to sustain hydration of aerial photosynthetic tissues.

Evolutionary origins and diversification of food and water transport systems

Studies based on fossil records and extant (living) bryophytes have established that developmental programs evolved to form specialized water and nutrient conducting tissues. Based on the fossil record, early pretracheophyte land plants appeared to have developed simple water-conducting conduits having smooth walls with small pores, likely derived from the presence of PD. Similar structures are present, for example, in some of the mosses, the most ancient being termed water-conducting cells (WCCs) and the more advanced being the hydroids of the peristomate mosses (Mishler and Churchill 1984; Kenrick and Crane 1997; Ligrone et al. 2012).

Hydroids often form a central strand in the gametophyte stem/sporophyte seta in the mosses (**Figure 1A, B**). During their development, these hydroid cells undergo various structural modifications to the cell wall and are dead at maturity (**Figure 1C, D**). Although in some cases the hydroid wall may become thickened, these are considered to be primary in nature and lack lignin. However, recent studies have indicated that bryophyte cell walls contain lignin-related compounds, but these do not impart mechanical strengthening properties (Ligrone et al. 2012). Although this absence of mechanical strength served as an impediment to an increase in body size, it allowed for hydroid collapse during tissue desiccation, and rapid rehydration following a resupply of water (**Figure 1E, F**), a feature that likely minimized cavitation of these WCCs (Ligrone et al. 2012) (see also later section). This trait may also have allowed peristomate mosses to expand into dryer habitats. The evolution of hydroids could have involved modification of existing WCCs. However, based on the distribution of WCCs in the early land plants (**Figure 2**), it seems equally probable that they arose through an independent developmental pathway after the loss of perforate WCCs (Ligrone et al. 2012).

The fossil record contains less information on the evolution of specialized food-conducting cells (FCCs), due in large part to their less robust characteristics that limited effective preservation. However, insights can be gained from studies on extant bryophyte species. As with WCCs, early FCCs were represented by files of aligned elongated cells in which the cytoplasmic contents underwent a series of positional and structural modifications (**Figure 3**). Here, we will use moss as an example; in some species (members of the order Polytrichales),

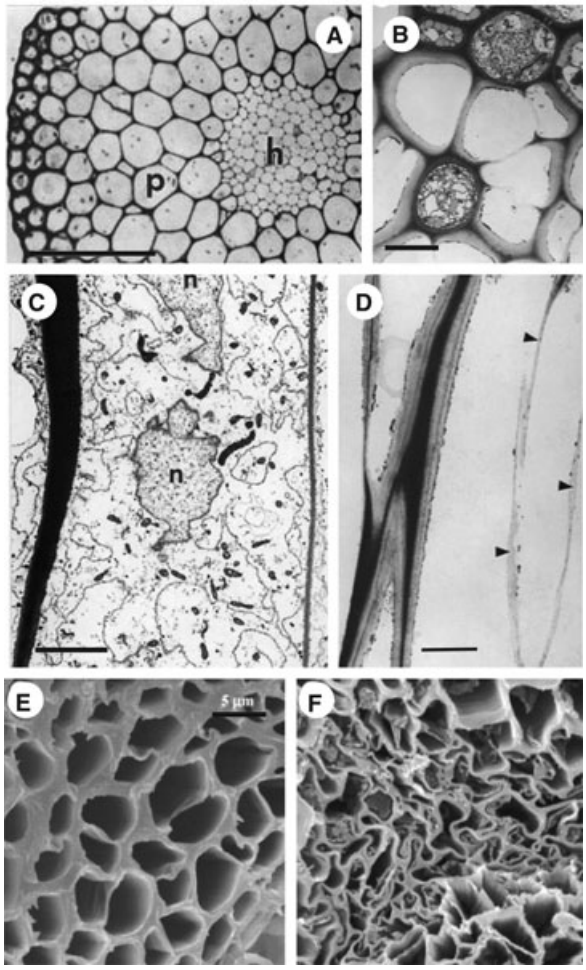

Figure 1. Water-conducting cells of early non-vascular (pre-tracheophyte) land plants.

(A) Light micrograph of a leafy stem from the moss *Plagiomnium undulatum* illustrating the prominent central strand of hydroid cells (h) surrounded by parenchyma cells (p).
(B–D) Transmission electron micrographs illustrating hydroids having unevenly thickened walls in the leaf shoot of the moss *Polytrichum juniperinum* **(B)**, a differentiating hydroid **(C)** and mature hydroids **(D)** in the leafy shoot of *Polytrichum formosum.*
(E, F) Transverse sections of moss hydroids in the hydrated **(E)** and dehydrated condition **(F)**; note that in the presence of water, dehydrated hydroids become rehydrated and functional. Images **(A–D)** reproduced from Ligrone et al. (2000), with permission of The Royal Society London; **(E, F)** reproduced from Ligrone et al. (2012), with permission of Oxford University Press. Scale bars: 50 μm in **(A)**, 5 μm in **(B–D)** and 5 μm in **(E)**, common to **(F)**.

the FCCs gave rise to a group of more specialized cells, termed leptoids and associated specialized parenchyma cells. During development, leptoids undergo a series of cytological changes, including cytoplasmic polarization and microtubule-associated alignment of plastids, mitochondria and the endoplasmic reticulum (ER), in a longitudinal pattern. At maturity, the FCCs of the bryophytes generally lack a large central vacuole and, in some species, there is partial degradation of the nucleus. In addition, the end walls of cells within these files of aligned FCCs develop a high density of PD (**Figure 4**), presumably to optimize symplasmic continuity for cell-to-cell diffusion of photosynthate (Ligrone et al. 2000, 2012; Raven 2003). Finally, FCCs/leptoids often develop in close proximity to WCCs/hydroids; in some species, the WCCs/hydroids are centrally located in the tissue/stem being ensheathed by FCCs/leptoids (**Figure 3E**).

As with hydroids, the evolutionary events leading to the development of FCCs, and leptoids in particular, appear to have been driven by the necessity to withstand periods in which the early land plants underwent desiccation. Insight sinto the presence and importance of such traits were offered by recent physiological and anatomical studies performed on the desiccation-resistant moss *Polytrichum formosum.* Here, the unique resilience of the lepoids to an imposed dehydration was shown to be associated with the unique role of microtubules in control over the special cytological features of these FCCs (Pressel et al. 2006). The properties of cavitation-resistant hydroids and desiccation-tolerant leptoids would likely have had an important impact on these mosses in terms of the ability to penetrate into diverse ecological niches.

Emergence of xylem with lignified tracheids and vessels

As indicated in **Figure 2**, xylem tissues may well have evolved independently from WCCs/hydroids. Although hydroids have a number of similar features to the early tracheary elements, including functioning after death, there are many important differences. Perhaps the most critical was the acquisition of a developmental program for the deposition of patterned secondary cell wall material. Of equal importance was the development of lignin and its deposition within the secondary wall of tracheary cells. Collectively, these evolutionary events imparted biomechanical support and compressive strength, with an ability to withstand tracheid collapse when the water column was placed under tension (see later section). Acquisition of biomechanical strength afforded the opportunity for an increase in plant height, with the benefit of enhanced competition for sunlight.

A further defining feature of the early vascular plants was that their tracheary (water conducting) elements had pits of varying architecture that spanned the secondary wall. In contrast to the WCCs of the bryophytes, the formation of these pits is not dependent upon the dissolution of PD (Barnett 1982; Lachaud and Mauroussset 1996), and in the early tracheary element, the tracheid (Edwards et al. 1992), the primary cell wall remains

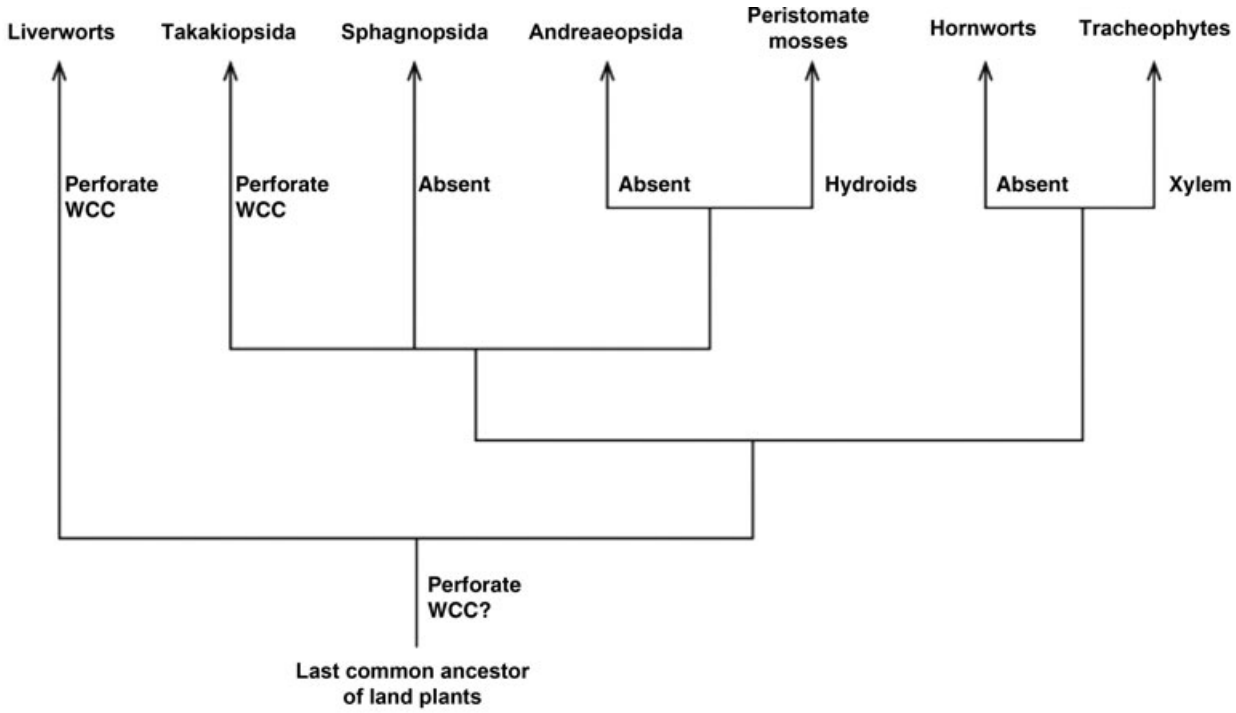

Figure 2. Cladogram illustrating the distribution of water-conducting cells (WCCs) in early land plants.

Note that hydroids in the peristomate mosses lack perforate cell walls. Reproduced from Ligrone et al. (2012), with permission of Oxford University Press.

imperforate. However, in the advanced form, the vessel element or vessel member, the primary wall is removed in discrete regions between adjacent members, thereby giving rise to a perforation plate. This evolutionary adaptation allows water to flow through many mature vessel members that collectively form a vessel, unimpeded by the primary cell wall; i.e., the perforation plate reduces the overall resistance to water flow through vessels.

Evolutionary relationship between FCCs and early tracheophyte sieve elements

The cytological features of FCCs are widespread in the bryophytes and many are also present in the phloem sieve elements of the lycophytes, pterophytes and gymnosperms (Esau et al. 1953) (Table 1). It is also noteworthy that the ER is present in PD located in the adjoining transverse walls between FCCs, leptoids and the sieve elements of ferns (Evert et al. 1989) and conifers (Schulz 1992). Furthermore, both leptoids and early sieve elements, termed sieve cells, have supporting parenchyma cells. These features, held in common between the more advanced FCCs and the phloem sieve elements of the early tracheophytes, raise the possibility of a developmental program having components shared between these nutrient delivery systems of the plant kingdom.

Evolution of molecular mechanisms regulating vascular development

Significant progress has been made in elucidating the molecular mechanisms regulating vascular development. In most cases, a modest number of angiosperm model species have been the focus of molecular-genetic and genomic analysis of vascular development. At present, individual genes regulating specific aspects of vascular development have been characterized in detail. In addition, models of how vascular tissues are initiated, patterned, balance proliferation and differentiation, and acquire polarity have been developed.

Vascular development is currently being modeled at new levels of complexity in *Arabidopsis* and *Populus*, using computational and network biology approaches that make use of extensive genomic gene expression and gene regulation datasets. While incomplete, new models representing important phylogentic positions in land plant evolution are also being developed, and will provide important insights into the origins and diversification of mechanisms regulating vascular development. Importantly, many of the key gene families that regulate vascular development predate tracheophytes. Thus, one major challenge for understanding the evolution of vascular development will be to determine the evolutionary processes by which

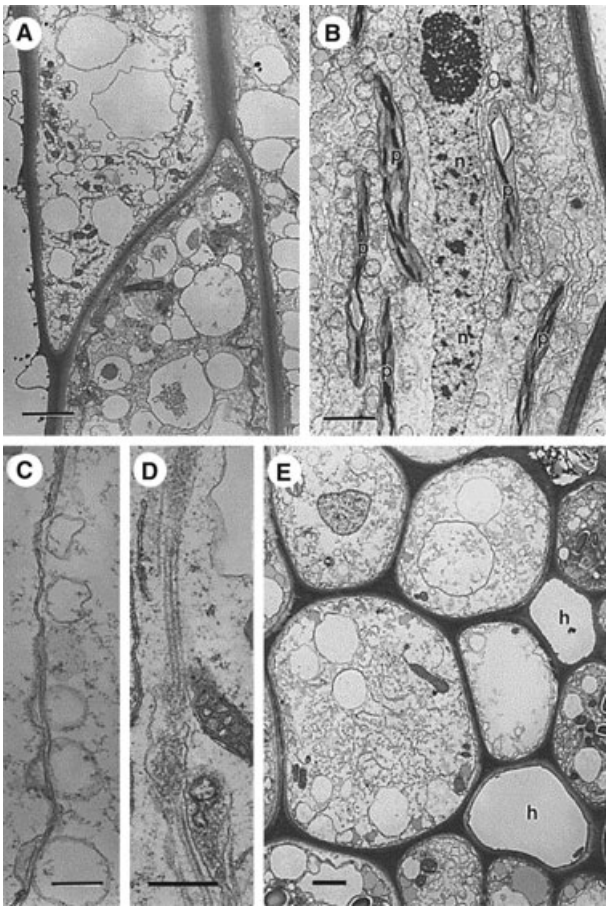

Figure 3. Cytological details of moss food-conducting cells.

(A) Cytoplasmic polarity in a leafy stem of *Plagiomnium undulatum*; most of the organelles are in the top end of the lower cell.
(B) Sphorophyte seta of *Mnium hornum* showing the longitudinal alignment of elongated plastids (p) and the highly elongated nucleus (n).
(C, D) Longitudinal arrays of microtubules associated with tubules and vesicles in a leafy stem of *Plagiomnium undulatum* **(C)** and *Polytrichum juniperinum* **(D)**.
(E) Transverse section of leptoids and adjacent hydroids (h) in a stem of *Polytrichum commune*.
Scale bars: 4 μm in **(A)**, 2 μm in **(B, E)**, 0.5 μm in **(C, D)**.
Reproduced from Ligrone et al. (2000), with permission of The Royal Society London.

regulatory genes and modules were duplicated, modified, or directly co-opted to function in vascular development (Pires and Dolan 2012). Even more challenging will be determining the evolutionary steps underlying the many biochemical processes required for the production of vascular tissues and lignified secondary cell walls.

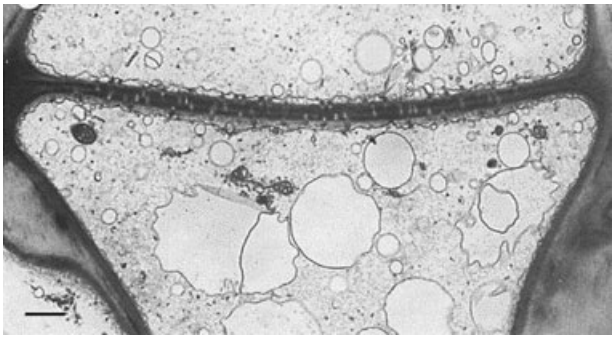

Figure 4. Abundant plasmodesmata in the trumpet-shaped end walls between food-conducting cells in the moss *Sphagnum cuspiatum*.

Scale bar: 10 μm. Reproduced from Ligrone et al. (2000), with permission of The Royal Society London.

Table 1. Comparison of cytological features present in moss food-conducting cells and sieve cells in ferns and conifers

Similarities[a]	Differences (in sieve cells)
Absence of vacuoles	No cytoplasmic polarization
Nacreous walls[b]	Apparent lack of polyribosomes
Nuclear degeneration[b]	
Presence of endoplasmic reticulum (ER) within plasmodesmata (PD)	
Callose associated with PD[c]	

[a]Modified after Ligrone et al. (2000).
[b]Restricted to the Polytrichales in mosses.
[c]Restricted to the Polytrichales in mosses, absent in some lower tracheophytes.

Auxin is an evolutionarily ancient regulator of vascular development

In the following sections we present some examples of the genes and mechanisms regulating specific aspects of vascular development. This is not a complete review of the literature, but rather we aim to highlight some of the molecular-genetic models of vascular development. We begin with the enigmatic plant hormone auxin, which has been known to play fundamental roles in vascular development for decades, but only recently have insights been gleaned at the molecular-genetic level as to how it exerts its many influences on vascular development. To understand the myriad of ways that auxin influences plant development, it is necessary to understand its synthesis, conjugation, transport, perception, and effects on gene expression. Fundamental insights into all of these processes have been gained, and have been summarized

in recent reviews. Importantly, auxin can apparently be synthesized by all plants (Johri 2008; Lau et al. 2009) in which it plays various roles in promoting growth, and has thus been recruited to participate in vascular development in the tracheopytes. Here, we consider one specific role for auxin during vascular development: how auxin transport proteins act during the establishment and propagation of interconnected vascular strands.

Vascular strands consist of interconnected files of cells. It is critical that vascular strands be properly spaced and patterned within tissues, and that functional cell types (e.g. water conducting tracheary elements) coordinate differentiation to produce interconnected conduits for transport. Auxin has long been known to play fundamental roles in both the induction of vascular strands and in the differentiation of vascular cell types (Sachs 1991). How auxin is transported through tissues has been recognized as a primary factor in determining the location and propagation (or canalization) of vascular strands. Major insights into how auxin transport is regulated have been gained through the identification and characterization of PIN-FORMED (PIN) proteins, which include plasma membrane spanning auxin efflux carriers. PIN proteins act through asymmetric subcellular localization to direct routes of auxin flow through cells and tissues (Petrášek et al. 2006, Petrášek and Friml 2009).

Importantly, PIN proteins are found in all land plants (Krecek et al. 2009) including the pretracheophytes, and polar auxin transport (PAT) already existed in the Charophyta (Boot et al. 2012), suggesting that ancestral functions of PIN proteins were not in vascular development, *per se*. The fossil record also provides insights into the role of auxin transport in the evolution of vascular tissues. Routes of auxin transport can be indirectly inferred from anatomical changes in extant angiosperms and gymnosperms, in which circular patterns of tracheary element develop in secondary xylem above branches, which impede auxin transport. Amazingly, fossils of wood from 375-million-year-old *Archaeopteris* (a progymnosperm) also show this pattern (Rothwell and Lev-Yadun 2005).

A general expectation is that the auxin-related mechanisms regulating vascular differentiation are shared (i.e. are homologous) among vascular plants, but this remains to be verified through functional studies in all the major vascular plant lineages. Perhaps more intriguing will be the characterization of ancestral auxin-related mechanisms in non-vascular plants and the determination of the evolutionary steps through which they were co-opted and modified during vascular plant evolution.

CLASS III HD-ZIP transcription factors

Gene transcription is a major mechanism for regulating vascular development. One increasingly well characterized transcriptional module is defined by the Class III homeodomain-leucine zipper (HD-ZIP) transcription factors. The genes encoding these transcription factors are evolutionarily ancient, and are found in all land plants (Floyd et al. 2006). Interestingly, transcript levels of Class III HD ZIPs are negatively regulated by miRNAs which are also highly conserved (Floyd and Bowman 2004). The Class III HD-ZIP gene family expanded and diversified in land plant lineages, acquiring new expression patterns and functions along the way. Indeed, the functions of Class III HD-ZIPs in regulating vascular tissues are undoubtedly derived, since Class III HD-ZIPs predate the appearance of vasculature in land plant evolution.

In *Arabidopsis*, the Class III HD-ZIP gene family is comprised of five genes, *REVOLUTA* (*REV*), *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *ATHB8*, and *CORONA*/*ATHB15*. Phylogenetic and functional relationships among them support the conclusion that *ATHB8* and *ATHB15*, and *REV*, *PHAB*, and *PHAV* represent subclades (Prigge et al. 2005, 2006; Floyd et al. 2006). Functional relationships among the Class III HD-ZIPs are complex, and different family members have been implicated in shoot apical meristem formation, lateral organ initiation, embryo patterning, and leaf polarity (Floyd et al. 2006). However, all five *Arabidopsis* Class III HD-ZIPs have been implicated in some aspect of vascular development. The role for the Class III HD-ZIPs in regulating vascular polarity will be discussed in depth later in the review.

The interplay of transcription and hormones is just one of the many areas ripe for exploration in terms of the evolution and development of vascular biology. Powerful new tools are available or quickly being developed that will result in dramatic changes to both the scope and level of complexity that can be addressed in future studies. For example, new sequencing technologies now allow for comprehensive cataloguing of gene expression in vascular tissues from virtually any species. These and related genomic technologies are also being used to provide massive datasets for new computational approaches, including network biology, which can model the complex interactions of genes that together regulate fundamental features of vascular development. Importantly, these new technologies must be integrated within the framework of paleobotany, plant anatomy, and plant physiology to provide meaningful models of the evolutionary steps that occurred, at the molecular-genetic level, to provide the diversity of vascular biology that we see in extant plants.

Phloem Development & Differentiation

Recently, several novel regulatory mechanisms that control the specification of vascular patterning and differentiation have been uncovered. Through the use of novel genomics and molecular techniques in several model plant systems, such as *Arabidopsis*, *Populus* and *Zinnia*, new insights have become

available regarding the regulation of vascular development. The importance of signaling in the control of vascular morphogenesis has become increasingly apparent. The primary agents involved include well-known phytohormones such as auxin, cytokinin and brassinosteroids, as well as other small regulatory molecules. This level of understanding also involves the transporters and receptors of these factors. There is increasing evidence for the notion that xylem and phloem development are highly coordinated. While many of the factors which will be described in this section of the review act cell-autonomously, the emerging importance of mobile regulatory factors will also be highlighted.

Embryonic provascular and shoot vascular development

During embryogenesis, the progenitor cells that will eventually become the vascular tissues are first established as undifferentiated procambial tissues. Surrounded by the epidermal and ground tissue layers, this procambial tissue forms the innermost domain of the plant embryo. By the late globular embryonic stage, the four procambium cells have undergone periclinal divisions to generate the future pericycle and the vascular primordium. The complete root promeristem with all initials and derived cell types is contained already in the early torpedo stage embryo (Scheres et al. 1995). Asymmetric cell divisions within the vascular primordium go on to establish the number of vascular initials present in the seedling root meristem.

In the aerial parts of a seedling, the protovascular elements are first specified and start to differentiate in the cotyledons and, only subsequently, in the axis (Bauby et al. 2007). Protophloem differentiation can first be observed in the midvein by the typical cell elongation, followed by extension to the distal loops and cotyledonary node. Before vasculature differentiation, continuous procambial strands can already be observed (**Figure 5**). The main agent in the establishment of this pattern is the phytohormone auxin. The accumulation of auxin, through polar transport mechanisms, as shown by the synthetic auxin reporter DR5 and the auxin-induced pre-procambial marker AtHB8, precedes the formation of vascular strands in leaves (Scarpella et al. 2004). Facilitating this highly localized auxin accumulation is the auxin transporter PIN1, which channels auxin to the provascular regions. PIN1 is already expressed before procambium formation.

After the initial differentiation, the vasculature develops in bundles (Esau 1969). These bundles have a very distinct radial pattern of abaxial phloem and adaxial xylem divided by an active procambium (**Figure 6**). The radial patterning of the vascular bundles is already established during embryogenesis by the main factors of radial patterning, KANADI (KAN) and the Class III HD-ZIPs, PHB, PHV, REV and CORONA/ATHB15 (McConnell et al. 2001; Emery et al. 2003). Auxin also plays a role in this determination of the radial vascular patterning (Izakhi and Bowman 2007). PIN1 localization is affected in *kan* mutants, showing the integration of the auxin transport pathway and KAN signaling. The triple mutant *phb phv rev* has radialized as well as abaxialized leaves and vascular bundles. In contrast, gain-of-function Class III HD-ZIP mutants with faulty microRNA (miRNA) regulation and *kan1 kan2 kan3* have radialized and adaxialized leaves and bundles. In addition, the Class III HD-ZIP genes also appear to regulate vascular tissue proliferation.

As will be described in more detail later, the phloem translocation stream, or phloem sap, contains not only photosynthate but also a wide array of macromolecules, such as mRNA, small RNAs and proteins. Both in animals and plants, small RNAs have already been identified as important regulatory factors controlling cell fate. A bidirectional cell-to-cell communication network involving the mobile transcription factor SHORTROOT (SHR) and microRNA165/166 species specifies the radial position of two types of xylem vessels in *Arabidopsis* roots (Carlsbecker et al. 2010; Miyashima et al. 2011). Since microRNA165/166 is a factor restricting PHB activity, it also regulates phloem development.

Recent studies have shown that CALLOSE SYNTHASE 3 (CALS3), a membrane-bound enzyme which synthesizes callose, a β-1,3-glucan (Verma and Hong 2001; Colombani et al. 2004), appears to be involved in regulating the cell-to-cell movement of microRNA165/166 (Vatén et al. 2011). CALS3 is expressed both in phloem and meristematic tissues. Gain-of-function mutations in CALS3 result in increased accumulation of PD callose, a decrease in the PD aperture, multiple defects in root development, and reduced intercellular trafficking of various molecules. Using an inducible expression system for a modified version of CALS3 (CALS3m), Vatén et al. (2011) were able to show that increased callose deposition inhibited SHR and microRNA165 movement between the stele and the endodermis. This interesting result suggested that regulated callose biosynthesis, at the PD level, may be essential for control over cell-to-cell communication and cell fate determination.

Root vascular development

In the root, protophloem initially differentiates from an independent differentiation locus in the upper hypocotyl; only later does protophloem differentiation from the root apical meristem begin. As the root grows, the cellular pattern is established and maintained by the self-renewal of pluripotent root meristem cells. Different cell identities are initiated from the stem cells around the quiescent center (QC): the provascular initials of the stele, the cortex/endodermal initials, the epidermal/lateral root initials, and the columella initials. In the *Arabidopsis* root, a central vascular cylinder (consisting of xylem, phloem and procambium) is surrounded by radially symmetric layers of pericycle, endodermis, cortex and epidermal cells. In this root

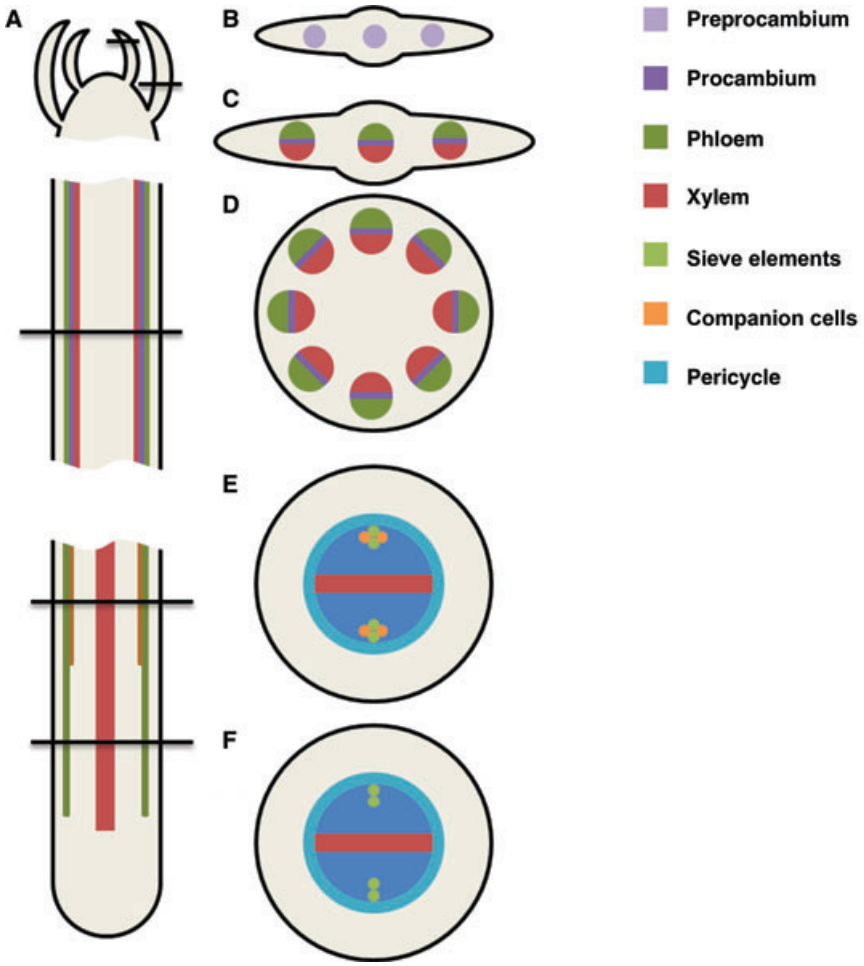

Figure 5. Schematic of the primary phloem organization in *Arabidopsis* shoot and root.

(A) Longitudinal section through the shoot apex, shoot and root.
(B) Cross section of an early developing leaf showing the preprocambial bundles which precede the vascular bundles.
(C) Cross section of a leaf showing established vascular bundles where primary phloem and xylem differentiate asymmetrically from a separating layer of procambium.
(D) Cross section of the stem showing primary vascular bundles.
(E) Cross section of the root showing the primary vascular patterning with two phloem poles consisting of sieve elements and companion cells flanking the xylem axis.
(F) Cross section of the root tip showing two poles of protophloem sieve elements flanking the xylem axis.

system, the vascular tissue is comprised of a central axis of water-conductive xylem tissue that is flanked by two poles of photoassimilate-conductive phloem tissue.

In the root apical meristem, the phloem cell lineages arise from two domains of initials through asymmetric cell divisions (Mähönen et al. 2000). Periclinal divisions establish companion cells (CCs) and tangential divisions establish sieve elements (SEs). This asymmetry allows these initials to give rise to multiple cell lineages with different fates; in addition to the phloem lineage, they also precede undifferented procambial cell lineages. As opposed to the invariant pattern of cell lineages in the endodermis and outer layers, the number and exact pattern of these procambial divisions vary between individual seedlings.

Mähönen et al. (2000) have described these initial asymmetric cell divisions in great detail through sequential cross-sections of the region immediately above the quiescent center, allowing the precise determination of the first true phloem domains. At first, although cell divisions and early xylem specification can be observed (though not yet the complete

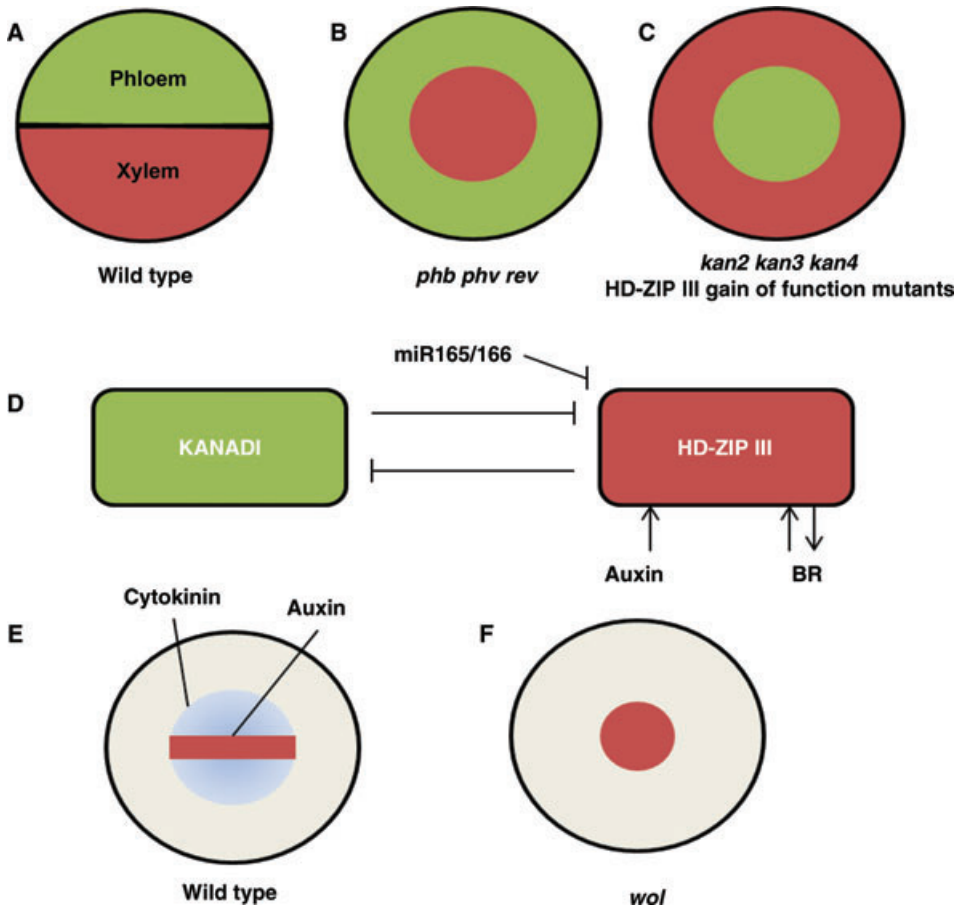

Figure 6. Vascular patterning is regulated by KANADI and Class III HD-ZIP genes and the phytohormones auxin and cytokinin.

(A) In shoot vascular bundles, the default radial pattern has phloem located abaxially and xylem adaxially.
(B) In the *phb phv rev* mutant, phloem surrounds xylem.
(C) Conversely, in Class III HD-ZIP gain-of-function mutants and *kan1 kan2 kan3*, xylem surrounds phloem.
(D) Class III HD-ZIP genes are regulated by miR165/166 and interact with auxin and brassinosteroids.
(E) In the root, auxin is restricted to the xylem axis by the presence of cytokinin.
(F) In mutants with defective cytokinin signaling such as *wol*, auxin is abundant throughout the stele, leading to ubiquitous protoxylem differentiation and loss of phloem identity.

xylem axis), phloem identity is not observable directly above the QC. The first newly formed cell walls associated with phloem development are only visible 27 μm above the QC. At a distance of 69 μm, the first protophloem SEs are clearly present.

Hormonal balance determines the development of vascular poles in the root

Cytokinin, an essential phytohormone for development in the root, is required for vascular patterning and the differentiation of all cell types except the protoxylem. Recently, it has been shown that the root vascular pattern is defined by a mutually inhibitory interaction between cytokinin and auxin (Bishopp et al. 2011a, 2011b). If cytokinin signaling is disturbed, as in the *WOODEN LEG* mutant, *wol* or the triple cytokinin receptor mutant *ahk2 ahk3 ahk4* (*ARABIDOPSIS HISTIDINE KINASE*), or if cytokinin levels are reduced, as is found in transgenic plants overexpressing CYTOKININ OXIDASE, the effect is always an increased number of protoxylem cell files and the loss of other cell types in the root vasculature. The domain of cytokinin activity is restricted by the action of the cytokinin signaling inhibitor, ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6). Only through this mechanism does protoxylem differentiation occur in a spatially specific manner, allowing for the proper development of the phloem cell types.

Phloem differentiation

Sieve elements comprise the main conductive tissue of the phloem. Like the CCs, they originate from phloem precursor cells in the procambium. However, very early in primary phloem development, they undergo dramatic changes in their morphology. As the SEs mature, they experience extensive degradation of their organelles. The nucleus, vacuoles, rough endoplasmic reticulum (ER) and Golgi are degraded in a process which has not yet been characterized at the molecular level. This reduction in cellular contents establishes an effective transport route through the sieve tubes. However, the SEs still remain living, as they retain a plasma membrane and a reduced number of other organelles, such as smooth ER, plastids and mitochondria. The residual ER is localized near the PD which interconnect the SEs to their neighboring CCs.

The cell walls of the SEs also undergo drastic changes in structure. The first observable process is an increase in callose that is deposited in platelet form around the PD of the SEs, replacing the already present cellulose. The cell walls which form the interface to adjoining SEs contain a high density of these callose-ensheathed PD. As these SEs mature, both these callose deposits and the middle lamella in these regions of the cell wall are removed, thereby forming a sieve plate with enlarged pores (Lucas et al. 1993). The lateral cell walls of SEs also develop specialized areas of PD-derived pores, which are called lateral sieve areas. Recent studies have shown that CALS3 and CALS7 are involved in depositing PD-callose during this developmental process (Vatén et al. 2011; Xie et al. 2011). The newly formed sieve plates, in combination with the lateral sieve areas, enable each individual SE to become a component of an integrated sieve tube system that can facilitate effective fluid transport by bulk flow. It is also noteworthy that these pores increase considerably in size as tissues age, thus increasing the transport potential of the more mature vasculature (Truernit et al. 2008).

The survival and differentiation of SEs depends on a close association with their neighboring CCs, a specialized type of parenchyma cell. The cytoplasm of the CC is unusually dense, due in part to an increased number of plastids, mitochondria and free ribosomes (Cronshaw 1981). The CCs are connected to their adjacent SEs by numerous branched PD. Through these connections, the enucleate SEs are supplied with energy, assimilates and macromolecular compounds, such as proteins and RNA (Raven 1991; Lough and Lucas 2006). The size exclusion limit of these PD connections usually lies between 10 and 40 kDa (Kempers and van Bel 1997), giving credence to the concept of protein transport from CCs to SEs.

The morphological and physiological uniqueness of the phloem cell types described above is also a result of specific gene expression patterns, as shown by recent transcriptome studies (Lee et al. 2006; Brady et al. 2007, 2011). These transcriptional programs are exquisitely controlled in space and time. To understand how these unique cell identities are acquired, a deeper understanding of these programs is absolutely essential. Microarray analyses of a high-resolution set of developmental time points, and a comprehensive set of cell types within the root, has resulted in the most detailed root expression map to date.

Numerous distinct expression patterns have been identified through these analyses, several of which are specific to SEs or to SEs and CCs together. More than a thousand genes have been identified as having phloem-specific expression, highlighting the phloem as a highly specialized tissue within the stele. The data from these microarray studies has been made publicly available in the AREX LITE: The Arabidopsis Gene Expression Database (arexdb.org), providing an invaluable resource for future studies on phloem function.

Bauby et al. (2007) identified several phloem markers, which they named Phloem Differentiation 1–5 (PD1-PD5) by screening the Versailles collection of gene trap mutants for plant lines expressing the *uidA* reporter gene in immature vascular tissues. PD1-4 were restricted to protophloem cells, as determined by their cell shape. However, PD5 was expressed in both protophloem and metaphloem cells. Using these markers, these authors could track the onset of phloem development directly after embryogenesis. PD4 is expressed in the tips of leaf primordia some 3 days after germination (dag). Spreading from there, by 7 dag, PD4 has already traced out the entire future leaf vasculature. The first expression of PD1 and PD3 was detected 3 dag in the proximal protophloem of leaf primordia. These results establish that PD1 and PD3 are expressed during the differentiation of protophloem. PD4 and PD5 gene reporter-based expression was also detected in the location of the midveins and higher order veins before procambium differentiation, thereby defining the pre-patterning of the future veins.

Regulation of phloem differentiation

Currently, only two factors are known which specify phloem identity: ALTERED PHLOEM DEVELOPMENT (APL) and OCTOPUS (OPS). APL is a MYB coiled-coil transcription factor essential for the proper differentiation of both SEs and CCs (Bonke et al. 2003). Additionally, APL contributes to the spatial limiting of xylem differentiation, is expressed both in SEs and CCs, and has been shown to be nuclear localized. Loss of APL function has an extraordinary effect on the phloem, as can be observed in the loss-of-function mutant *apl1* (**Figure 7**). This mutant is seedling-lethal and results in short-rooted plants. Neither CCs nor SEs can be detected in cross-sections of *apl1* plants. Furthermore, phloem-specific reporters, such as the CC-specific sucrose transporter, SUC2, or the proto SE reporter, J0701, cease to be expressed entirely in these mutant

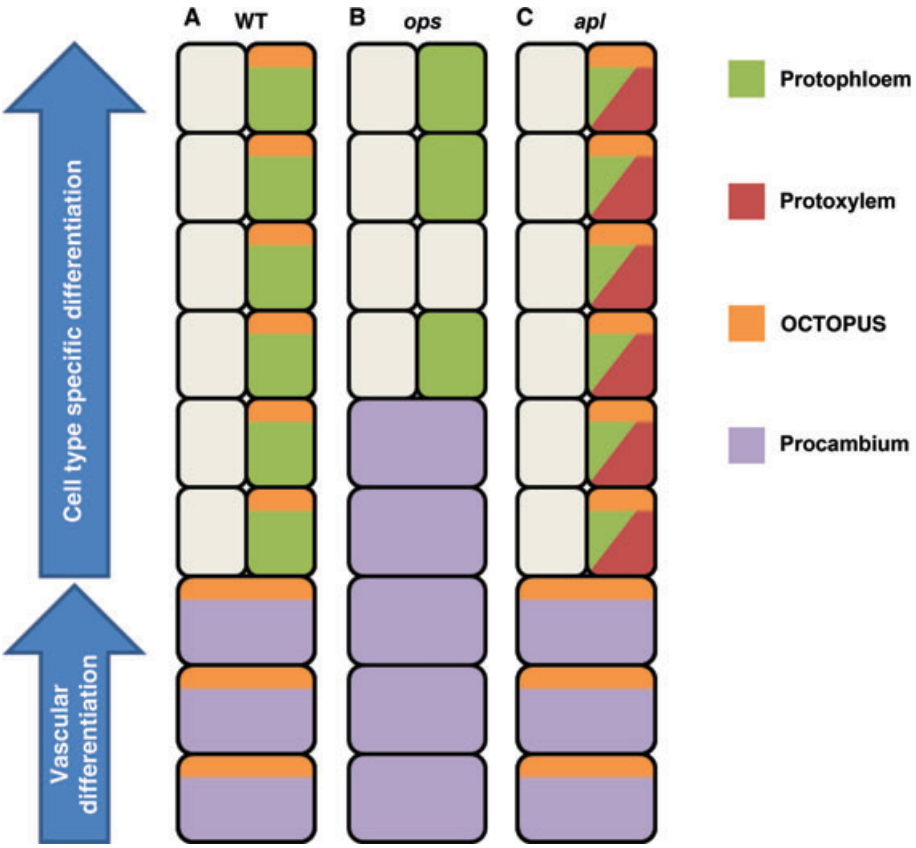

Figure 7. Model of OCTOPUS (OPS) and ALTERED PHLOEM DEVELOPMENT (APL) action.

(A) OPS is located at the apical plasma membrane in the procambium and phloem lineage. OPS interprets vascular signals for phloem differentiation, such as APL.

(B) In the *ops* mutant, phloem differentiation is delayed. Procambial cell number is increased and gaps of undifferentiated cells are visible in the protophloem strand.

(C) In the *apl* mutant, the initiation of phloem differentiation is largely unperturbed, however proper protophloem fails to emerge. In their place, protophloem/protoxylem hybrid cells appear.

plants. This dramatic effect is restricted to the phloem poles; other cell types appear similar to wild-type (WT) plants.

As mentioned above, asymmetrical cell divisions establish the phloem poles in wild-type plants; periclinal divisions establish CCs and tangential divisions establish SEs. In the *apl* mutant, these cell divisions are often delayed, but they still take place, so APL does not appear to be required for these asymmetric cell divisions. However, since the subsequent differentiation of SEs cannot be observed, it can be concluded that APL is responsible for phloem differentiation, rather than the establishment of the phloem cell lineage.

It has also been proposed that APL acts as an inhibitor to xylem differentiation. In the *apl* mutant, ectopic xylem strands are seen in the place of the phloem poles. When APL is expressed ectopically in vascular bundles, xylem formation is inhibited. Recently, novel imaging techniques were employed to analyze the *apl* mutant in more detail (Truernit et al. 2008). With this increased resolution, it was discovered that protophloem differentiation proceeds normally in this mutant until 2 dag. At this time, cells in the protophloem position display the normal characteristic shape and cell wall thickening. However, after this period, the previously described acquisition of xylem characteristics was observed, although the cells in place of the SEs still formed sieve plates. This finding suggests that these cells can be classified as hybrids between phloem and xylem (Truernit et al. 2008). Thus, APL is absolutely required for the later stages of phloem development, although it is not the earliest factor acting in this process. The identity of such a factor, or factors, has yet to be determined. Additional support for the presence of such factor(s) was provided by Lee et al. (2006), who pointed out that there are numerous phloem markers with earlier SE expression than APL.

In addition to *APL*, *OPS* has also been identified as a gene related to phloem cell differentiation; this gene is required for phloem continuity during phloem development (Truernit et al. 2012). Interestingly, OPS was first reported based on its vascular expression pattern (Nagawa et al. 2006). Further detailed analysis revealed that its expression is initially in the provascular cells at the heart stage of embryo development, and it subsequently becomes restricted to the phloem lineage cells following phloem cell-type specification (Bauby et al. 2007; Truernit et al. 2012). Unlike *APL*, *OPS* expression can be observed in the phloem and procambium initials near the QC.

Vascular patterning of the cotyledons, in the mature embryo of the *ops* mutant, is delayed, and the number of completed vascular loops in the developing cotyledon is reduced (Truenit et al. 2012). In contrast, the progression of vascular patterning is accelerated by OPS overexpression in the cotyledons of mature embryos, and the number of completed vascular loops in the developing cotyledon is increased. This suggests that OPS is involved in promoting the progression of vascular patterning. Furthermore, Truernit et al. (2012) found that, in *ops* hypocotyls and roots, the phloem SE cell file was interrupted by undifferentiated SEs, which failed to undergo an increase in cell wall thickness, callose deposition, or nuclear degradation; these cells failed to acquire SE-specific PD1 marker expression (Truernit et al. 2012). These cellular differentiation defects caused inefficient phloem transport in the root. These phenotypes indicated that OPS was required for continuous phloem development.

OPS encodes a membrane-associated protein (Benschop et al. 2007) specific to higher plants (Nagawa et al. 2006). The function of OPS is currently unknown; no functional domain has been identified in this protein. However, a functionally complementing OPS-GFP protein was located at the apical end of the SEs (Truernit et al. 2012). Inductive cell-to-cell communication from differentiated to undifferentiated neighboring cells is known to occur during xylem differentiation; XYLOGEN is a polar-localized proteoglycan-like factor required to direct continuous xylem differentiation in *Zinnia elegans* L. and *A. thaliana* (Motose et al. 2004). It is thought that, in a similar manner, OPS contributes to longitudinal signaling, thereby inducing SE differentiation in undifferentiated SE precursor cells. Further study of OPS function and identification of factors relating to OPS function will advance our understanding of how phloem continuity is organized and how the phloem develops.

The *Arabidopsis LATERAL ROOT DEVELOPMENT 3* (*LRD3*) is another important gene which has been reported to regulate early phloem development and to control transport function of phloem (Ingram et al. 2011). The *LRD3* gene encodes a LIM-domain protein which is specifically expressed in the CCs. The normal function of LRD3 is to maintain a balance between primary and lateral root growth and phloem-mediated resource allocation within the root system. The *lrd3* loss of function mutant has decreased primary and increased lateral root growth and density, without having a significant effect on sucrose uptake. Additionally, aniline blue staining of the *lrd3* primary root shows an overall reduction in the callose level in root meristems, developing SEs, and the PD that connect CCs to SEs, suggesting a non-cell-autonomous role for LRD3 in early phloem development.

A detailed analysis of long-distance transport using different transport assays, including ^{14}C-sucrose, the fluorescent tracer dye carboxyfluorescein diacetate (CFDA) and CC-driven GFP (*AtSUC2::GFP*), demonstrated that phloem translocation to the primary root tip is severely limited in young *lrd3* plants, whereas phloem loading and export from the shoot appear to be normal. Notably, these phloem defects were subsequently rescued, spontaneously, in older plants, along with a subsequent increase in phloem delivery to and growth of the primary root. Importantly, continuous exogenous auxin treatment could rescue the early phloem developmental defects and transport function in the primary roots of *lrd3*. This finding suggested either that auxin functions downstream of LRD3, or that it may have an independent key role in early phloem development. Interestingly, this study of the effects of *lrd3* on root system architecture and the pattern of phloem translocation in the root system suggests that there might be some tightly regulated mechanism(s) which selectively supports a biased phloem-mediated resource allocation in the lateral roots when the primary root is compromised.

Phloem: A conduit for delivery of photosynthate and information molecules

Phloem is an important organ, vital for more than just the well-established function of photoassimilate transport from the photosynthetic organs to the sink tissues. New transport functions continue to be discovered, such as the phloem-based transport of phytohormones, small RNAs, mRNAs and proteins. As will be discussed later, this transport of macromolecules appears to play a role in facilitating the coordinated developmental programs in meristematic regions located at various locations around the body of the plant.

In addition to its specialized transport functions, phloem also stands out by the highly distinctive morphology of its cell types. In the angiosperms, the interaction between the enucleate SEs and the CCs needs to be highly integrated in order to maintain the operation of the sieve tube system (van Bel 2003). This process likely involves the cell-to-cell trafficking of a wide range of molecules via the PD that interconnect the SE-CC complex. Future studies on the nature of this molecular exchange will be greatly assisted by the use of the modified CALS3m system (Vatén et al. 2011) which can serve as an effective tool to modulate transport between specific cell types.

Molecular Mechanisms Underlying Xylem Cell Differentiation

In the shoot apical meristem, stem cells differentiate into various cell types that comprise the shoot, while still proliferating in order to maintain themselves (Weigel and Jürgens 2002). Similarly, in the vascular meristem, procambial and cambial cells differentiate into specific vascular cells, such as tracheary elements, xylem fiber cells, xylem parenchyma cells, SEs, CCs, phloem parenchyma and phloem fiber cells, while again maintaining activity to proliferate (**Figure 8**). Therefore, procambial and cambial cells are considered as vascular stem cells (Hirakawa et al. 2010, 2011; Miyashima et al. 2012). Recent studies have revealed that local communication between vascular stem cells and differentiated vascular cells directs the well-organized formation of vascular tissues (Lehesranta et al. 2010; Hirakawa et al. 2011). During this vascular formation, plant hormones, including auxin, cytokinin and brassinosteroids, act as signaling molecules that mediate in this process of cell-cell communication (Fukuda 2004). In addition, recently, a tracheary element differentiation inhibitory factor (TDIF), a small peptide, was found to function as a signaling molecule both inhibiting xylem cell differentiation from procambial cells and promoting procambial cell proliferation (Ito et al. 2006; Hirakawa et al. 2008, 2010). TDIF belongs to the CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) family, some of whose members are central players in cell-cell communication within meristems (Diévart and Clark 2004; Matsubayashi and Sakagami 2006; Fiers et al. 2007; Fukuda et al. 2007; Jun et al. 2008; Butenko et al. 2009; Betsuyaku et al. 2011).

Further insight into the differentiation of procambial cells into xylem cells has been gained from recent comprehensive gene expression and function analyses (Kubo et al. 2005; Zhong et al. 2006; Yoshida et al. 2009; Ohashi-Ito et al. 2010; Yamaguchi et al. 2011). In particular, the discovery of master genes that induce differentiation of various xylem cells greatly enhanced our understanding of xylem formation (Kubo et al. 2005; Zhong et al. 2006; Mitsuda et al. 2007). Further analysis of these master genes revealed transcriptional networks that control xylem cell differentiation, which involves specialized secondary wall formation. Tracheary element differentiation also involves programmed cell death (PCD) (Fukuda 2000). In this section of the review, we will evaluate advances in our understanding of xylem cell differentiation from procambial cells, with a focus on cell-cell signaling, the underlying transcriptional network and the onset of PCD.

Intercellular signaling pathways regulating xylem differentiation

The TDIF-TDR signaling pathway regulates vascular stem cell maintenance. TDIF is a CLE-family peptide composed of twelve amino acids with hydroxylation on two proline residues (Ito et al. 2006). In the *Arabidopsis* genome, this TDIF sequence is encoded by two genes, *CLE41* and *CLE44* (Hirakawa et al. 2008). The TDIF RECEPTOR/PHLOEM INTERCALATED WITH XYLEM (TDR/PXY) is a receptor for TDIF, which belongs to the Class XI LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASE (LRR-RLK) family (Hirakawa et al. 2008).

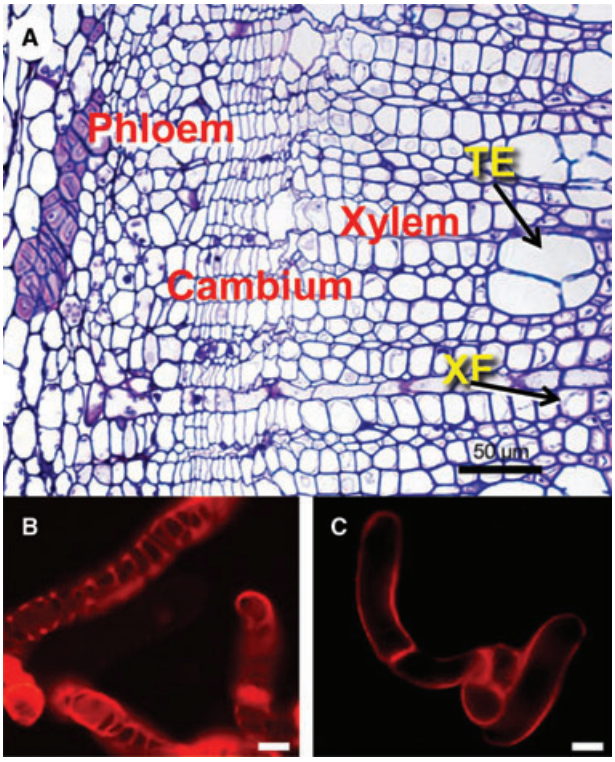

Figure 8. Xylem cells.

(A) Poplar vascular tissue in which tracheary elements (TE) and xylem fiber (XF) cells are formed.
(B) VND6-induced tracheary elements.
(C) SND1-induced xylem fiber cells.
Scale bars: 25 μm in **(B)** and **(C)**.

Genetic and physiological analyses have revealed that the TDIF-TDR signaling pathway is crucial for vascular stem cell maintenance, by inhibiting xylem differentiation from procambial cells and promoting procambial cell proliferation (Hirakawa et al. 2008) (**Figure 9**). *TDR* is expressed preferentially in the procambium and cambium (Fisher and Turner 2007; Hirakawa et al. 2008), whereas *CLE41* and *CLE44* are expressed specifically in the phloem and more widely in its neighbors, respectively. Defects in *TDR* or *CLE41* cause the exhaustion of procambial cells located between the phloem and xylem, resulting in formation of xylem vessels adjacent to phloem cells in the hypocotyl (Fisher and Turner 2007; Hirakawa et al. 2008, 2010). Ectopic expression of *CLE41*, under either a

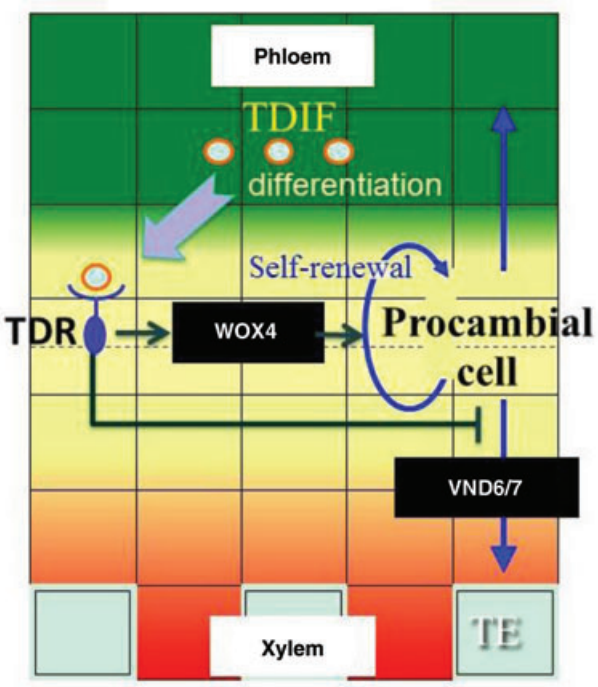

Figure 9. Regulation of procambial cell fates by the tracheary element differentiation inhibitory factor (TDIF) –TDIF receptor (TDR) signaling pathway.

TDIF is produced in phloem cells, secreted from phloem cells, and perceived by TDR in procambial cells. TDR signaling is diverged into two pathways: one promotes self-renewal via WOX4, and the other inhibits tracheary element (TE) differentiation from procambial cells, probably indirectly via the suppression of VND6/VND7.

xylem-specific or *35S* promoter, disrupts the normal pattern of vascular tissues, indicating the importance of the synthesis site for this signaling peptide (Etchells and Turner 2010). Thus, phloem-synthesizing TDIF regulates procambial cell fate in a non-cell-autonomous fashion.

The TDIF peptide signal activates expression of *WOX4,* a member of the *WUSCHEL-related HOMEOBOX* (*WOX*) gene family, in procambial and cambial cells (Hirakawa et al. 2010; Ji et al. 2010; Suer et al. 2011). Interestingly, *WOX4* is required for TDIF-dependent enhancement of procambial cell proliferation, but not for the TDIF-dependent suppression of xylem differentiation (Hirakawa et al. 2010). Ethylene/ERF signaling is reported to be another pathway to regulate procambial/cambial cell division and may function in parallel to the CLE41-TDR/PXY pathway, and, under normal circumstances, TDR/PXY signaling acts to repress the ethylene/ERF pathway (Etchells et al. 2012). Hence, at least two intracellular signaling pathways that diverge after TDIF recognition by TDR may regulate, independently, the behavior of vascular stem cells. Lastly, TDIF, which is produced mainly by CLE42, has also been shown to play a role in axillary bud formation in *Arabidopsis*, indicating that it is a multifunctional peptide signal in plants (Yaginuma et al. 2011).

CLE peptides inhibit protoxylem vessel formation through activating cytokinin signaling. Cytokinin is a key regulator of xylem development (Mähönen et al. 2000, 2006; Mok and Mok 2001; Matsumoto-Kitano et al. 2008; Bishopp et al. 2011b). Recent studies have revealed crosstalk between CLE peptide and cytokinin signaling, which regulates xylem differentiation (Kondo et al. 2011). In roots, TDIF does not significantly affect vascular development (Kondo et al. 2011). In contrast, treatment with some CLE peptides, including CLE9/CLE10, inhibits formation of protoxylem but not of metaxylem vessels in *Arabidopsis* roots. *CLE9* and *CLE10,* which encode the same CLE peptide, are preferentially expressed in vascular cells of roots (Kondo et al. 2011). Microarray analysis revealed that the CLE9/CLE10 peptide specifically reduces expression of type-A *ARABIDOPSIS RESPONSE REGULATERs* (*ARRs*) which are known as negative regulators of cytokinin signaling (Kiba et al. 2003; To et al. 2004, 2007).

The *ARR5* and *ARR6* are particular CLE9/CLE10 targets and, consistent with this finding, in the root of *arr5arr6* double mutant plants, protoxylem vessel formation is often inhibited (Kondo et al. 2011). Conversely, *arr10arr12,* a double mutant for two type-B ARRs, which function positively in cytokinin signaling, displayed ectopic protoxylem vessel formation. Furthermore, *arr10arr12* was resistant to the CLE9/CLE10 peptide in terms of protoxylem vessel formation. Interestingly, other combinations of type-B ARR mutants, such as *arr1arr12* and *arr1arr10*, showed much weaker resistance against the CLE9/CLE10 peptide compared with *arr10arr12*. This result implies that *ARR10* and *ARR12* act as major Type-B ARRs. Thus, the CLE9/CLE10 peptide activates cytokinin signaling through the repression of *ARR5* and *ARR6,* resulting in the inhibition of protoxylem vessel formation. Genetic analysis suggests that the CLV2 membrane receptor and its partner CRN/SOL2 kinase (Miwa et al. 2008; Müller et al. 2008) may act in protoxylem vessel formation, downstream of the CLE9/CLE10 peptide signaling (Kondo et al. 2011).

For cell-to-cell communication, plant cells send signaling molecules via the symplasmic pathway. A GRAS-family transcription factor, SHR, is a signal that moves cell to cell selectively through PD. SHR proteins are known to move from the stele into the endodermis to induce another GRAS-family transcription factor, SCARECROW (SCR), and then, together with SCR, they up-regulate expression of target genes, including the miR165/166 genes (Levesque et al. 2006; Cui et al. 2007; Gallagher and Benfy 2009). The mature miR165/166 moves back from the endodermis into the pericyle and protoxylem vessel poles in the stele, most likely through PD. Here, miR165/166 degrades the transcripts of *PHB* and its family of Class III HD-ZIP genes (Carlsbecker et al. 2010). These transcripts within

xylem precursors specify central metaxylem vessels, at high levels, and peripheral protoxylem vessels, at low levels. This reciprocal signaling between the inner vascular tissues and the surrounding cell layers allows the domain of response to be confined to one of two tissue compartments (Scheres 2010). Genome-wide analysis revealed the presence of direct targets of SHR not only in the endodermis but also in the xylem and pericycle, suggesting a complex function of this transcription factor in vascular development (Cui et al. 2012).

Thermospermine, a structural isomer of spermine (Ohsima 1979), has been shown to act as a suppressor of xylem development. ACAULIS 5 (ACL5) encodes a thermospermine synthase (Kakehi et al. 2008) that is expressed specifically in early developing vessel elements (Muñiz et al. 2008). ACL5 loss-of-function mutants cause excessive differentiation of xylem cells (Hanzawa et al. 1997; Clay and Nelson 2005; Muñiz et al. 2008). Exogenously applied themospermine suppresses xylem vessel differentiation in both *Arabidopsis* plants and a *Zinnia* xylogenic culture (Kakehi et al. 2010). Genetic analysis of *acl5* identified a suppressor of the *acl5* phenotype, *sac51*, whose causal gene encodes a basic helix-loop-helix (bHLH) transcription factor (Imai et al. 2006). Thermospermine is considered to regulate translational activity of *SAC51* mRNA, resulting in the suppression of xylem development (Imai et al. 2008). A recent chemical biology approach also indicated that the SAC51-mediated thermospermine signaling pathway can limit auxin mediated promotion of xylem differentiation (Yoshimoto et al. 2012). Thus, the possibility exists that ACL5 may control xylem specification through the prevention of premature cell death (Muñiz et al. 2008; Vera-Sirera et al. 2010).

Transcriptional regulation of xylem cell differentiation

The Class III HD-ZIP genes have been shown to regulate xylem differentiation. In the *phb phv rev cna athb8* mutant background, procambial cells fail to differentiate into xylem cells, but proliferate actively to produce many procambium cells. However, every quadruple loss-of-function mutant of the five Class III HD-ZIP genes exhibits ectopic xylem formation in the roots (Carlsbecker et al. 2010). In contrast, a gain-of-function mutant of *PHB* induces ectopic metaxylem vessel formation (Carlsbecker et al. 2010), and overproduction of *ATHB8* promotes xylem differentiation (Baima et al. 1995). These findings indicate that the Class III HD-ZIP members function positively in xylem specification. However, the regulation of xylem differentiation by these genes is more complicated. In roots, miR165/166, which degrades Class III HD-ZIP transcripts, promotes protoxylem vessel differentiation. Therefore, it is proposed that high transcript levels of these genes inhibit protoxylem vessel formation, but promote metaxylem vessel formation. Because exogenously applied brassinosteroids promote the expression of Class III HD-ZIP genes (Ohashi-Ito and Fukuda 2003) and xylem cell differentiation (Yamamoto et al. 1997), brassinosteroids may promote xylem differentiation, at least partly, through activation of these genes. These Class III HD-ZIP and KANADI transcription factors were also reported to regulate cambium cell differentiation, in which KANADI might act by inhibiting auxin transport and Class III HD-ZIPs by promoting xylem differentiation (Ilegems et al. 2010; Robischon et al. 2011).

Members of a subgroup of NAM/ATAF/CUC (NAC) domain proteins, namely the VASCULAR-RELATED NAC-DOMAINs (VNDs) and NAC SECONDARY WALL THICKENING PROMOTING FACTORs/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEINs (NSTs/SNDs), function as master transcription factors that can induce xylem cell differentiation by their ectopic expression (Demura and Fukuda 2007; Zhong and Ye 2007). VND6 and VND7 initiate metaxylem and protoxylem vessel differentiation, respectively (Kubo et al. 2005). Similarly, SND1/NST3 and NST1 induce xylem fiber differentiation (Mitsuda et al. 2005, 2007; Zhong et al. 2006). However, a single loss-of-function mutant of each gene shows no morphological defects, suggesting that other family members may have redundant functions to induce xylem differentiation, although each does not induce xylem cell differentiation when overexpressed (Kubo et al. 2005).

The activity of these master transcription factors appears to be regulated by the following three mechanisms. (1) Expressions of *VND7* and two genes for AS2/LBD domain-containing proteins, *ASL20*/*LBD18* and *ASL19*/*LBD30*, form a positive feedback loop to amplify their expression (Soyano et al. 2008). This rapid amplification of the master transcription factor may drive xylem cell differentiation promptly and irreversibly. (2) VND7 activity is also regulated at the protein level by its proteasome-mediated degradation (Yamaguchi et al. 2008). (3) A NAC domain transcription repressor, VND-INTERACTING2 (VNI2), represses VND activity by protein-protein interaction (Yamaguchi et al. 2010). VNI2 has an unstable property because of the PEST proteolysis target motif in its C-terminal region, which may allow VND7 to exert its function promptly when it is required.

Xylem cells form characteristic secondary walls. These morphological events are regulated by master regulators such as SND1/NST3, VND6 and VND7. Microarray experiments revealed that VND6, VND7 and SND1 induce a hierarchical gene expression network (Zhong et al. 2008; Ohashi-Ito et al. 2010; Yamaguchi et al. 2010). These master transcription factors induce, probably directly, the expression of genes for other transcription factors, such as *MYB46*, *MYB83*, and *MYB103*. MYB46 and MYB83 regulate redundant biosynthetic pathways for all three major secondary wall components, namely cellulose, lignin, and xylan (Zhong et al. 2007; McCarthy et al. 2009). Here, two NACs and 10 MYBs appear to act downstream of SND1 (Zhong et al. 2008; Zhou et al. 2009). Of them,

MYB58, MYB63 and MYB85, which might be target genes of MYB46 and/or MYB83, specifically upregulate genes related to the lignin biosynthetic pathway (Zhou et al. 2009). Some of these key transcription factors are also induced by VND6 and VND7, suggesting that this hierarchal structure is also true in the case of VND6 and VND7 (Ohashi et al. 2010; Yamaguchi et al. 2010). However, each master regulator also induces the expression of distinct genes, including transcription factors. Thus, SND1/NST3, VND6 and VND7, as master regulators, switch at the top of the hierarchy to upregulate transcription factors such as MYBs, which, in turn, functioning as the second and third regulators, upregulate expression of genes encoding enzymes catalyzing secondary wall thickening during specific stages of xylem cell differentiation.

Interestingly, VND6 and VND7 can directly upregulate the expression of genes for enzymes such as *XCP1* and *CESA4*, which are ranked lowest, as well as genes for transcription factors, such as *MYB46*, which are ranked higher in the gene expression hierarchy (Ohashi-Ito et al. 2010; Yamaguchi et al. 2011). Similarly, genes for enzymes such as *4CL1* are direct targets of SND1 (Zhong et al. 2008; McCarthy et al. 2009). These findings indicate a sophisticated transcriptional regulatory network, by the master regulator, over a hierarchy. Tracheary elements and xylem fibers possess different characteristics, such as cell wall structure and PCD. In accordance with these characters, VND6, but not SND1, induces the expression of genes related to rapid PCD, such as *XCP1* and *XCPs*, while SND1, but VND6 preferentially, upregulates genes for lignin monomer synthesis, such as *PAL1, 4CL3*, and *CCoAOMT* (Ohashi-Ito et al. 2010).

It is well-established that an 11-bp *cis*-element named the tracheary-element-regulating *cis*-element (TERE), which is found in upstream sequences of many genes expressed in xylem vessel cells, is responsible for xylem vessel cell-specific expression (Pyo et al. 2007). VND6 binds the TERE sequence and activates the TERE-containing promoter, *in planta*, but not a mutated promoter having substitutions in the TERE sequence (Ohashi-Ito et al. 2010). VND7 also binds TERE (Yamaguchi et al. 2011). These results demonstrate that TERE is one of the target sequences contained within the *VND6* promoter. In contrast, SND1 specifically binds to a 19-bp sequence named SECONDARY WALL NAC BINDING ELEMENT (SNBE), to activate its target genes (Zhong et al. 2010).

Cellular events underlying xylem cell formation

Xylem cell differentiation involves temporal and spatial regulation of secondary cell wall deposition. A number of xylem cell types exist, such as those with annular and spiral patterns in protoxylem vessels, reticulate and pitted patterns in metaxylem vessels, and a smeared pattern in xylem fibers. The cortical microtubules regulate the spatial pattern of the secondary cell wall by orientating cellulose deposition. By using cultures expressing GFP-tubulin it was discovered that cortical microtubules became gradually bundled, which, in turn, was followed by secondary wall deposition (Oda et al. 2005, 2006, 2010). It is important to find microtubule associated proteins (MAPs) involved in secondary wall formation and to know their function. In this context, some important secondary wall-related MAPs that regulate cortical microtubule orientation have been discovered. For example, AtMAP70 family proteins appear to be involved in the formation of the secondary wall boundary (Pesquet et al. 2010). The plant-specific microtubule binding protein MIDD1/RIP3 promoted microtubule depolymerization in the future secondary wall pit area, resulting in a secondary wall-depletion domain (Oda et al. 2010). Further analysis revealed that ROPGEF4 and ROPGAP3 mediate local activation of the plant Rho GTPase ROP11, and this activated ROP11 then recruits MIDD1 to induce local disassembly of cortical microtubules (Oda and Fukuda 2012b). Interestingly, and conversely, cortical microtubules eliminate active ROP11 from the plasma membrane through MIDD1. Such a mutual inhibitory interaction between active domains of ROP and cortical microtubules gives rise to distinct patterns of secondary cell walls. These findings shed new insights into the microtubule organizing mechanism regulating secondary wall patterning (Oda and Fukuda 2012a).

PCD is a genetically regulated cell suicide process involved in many aspects of plant growth, such as seed germination, vascular differentiation, aerechyma tissue formation, reproductive organ development and leaf senescence (Kuriyama and Fukuda 2002). During xylem development, rapid and slow PCD occurs in tracheary elements and xylem fiber cells, respectively, in order to facilitate the removal of cellular content for the formation of dead cells with secondary walls (Bollhoner et al. 2012). PCD during tracheary element differentiation has long been recognized as an example of developmental PCD in plants (Fukuda 2004; Turner et al. 2007). This process includes cell death signal induction, accumulation of autolytic enzymes in the vacuole, vacuole swelling and collapse, and degradation of cell contents followed by mature tracheary element formation (Fukuda 2000).

It has been suggested that the signals for xylem cell death are produced early during xylem differentiation, and that cell death is prevented through the action of inhibitors and the storage of hydrolytic enzymes in the vacuole (Bollhoner et al. 2012). According to the morphological process, the death of xylem tracheary elements is defined as a vacuolar type of cell death (Kuriyama and Fukuda 2002; Van Doorn et al. 2011a). Vacuolar membrane breakdown is the crucial event in tracheary element PCD, and bursting of the central vacuole triggers autolytic hydrolysis of the cell contents, thereby leading to cell death (Bollhoner et al. 2012).

Microarray analyses of gene expression have revealed a simultaneous expression of many genes involved in both secondary wall formation and PCD (Demura et al. 2002; Milioni et al. 2002; Kubo et al. 2005; Pesquet et al. 2005; Ohashi et al. 2010). As mentioned above, recent results have demonstrated that a transcriptional regulatory system, composed of transcription factors such as VND6 and a TERE-*cis* sequence, regulates the simultaneous expression of genes related to both secondary wall formation and PCD in tracheary elements (Ohashi-Ito et al. 2010). These findings indicate that tracheary element-differentiation-inducing master genes initiate at least a part of PCD directly by activating PCD-related genes through binding the TERE sequence in their promoters. This suggests that, in contrast to apoptosis in animals, in which a common intracellular signaling system induces PCD, in plants, various developmental processes involving PCD may be regulated independently involving their own specific developmental steps.

Nitric oxide (NO) and polyamine have been suggested as signals involved in the cell death induction in xylem development. NO production is largely confined to xylem cells; removal of NO from the cultured *Zinnia* cells, with its scavenger PTIO, results in dramatic reductions in both PCD and in the formation of tracheary elements (Gabaldon et al. 2005). Thus, NO might well be an important factor mediating PCD during tracheary element differentiation.

Execution of PCD in developing tracheary elements involves expression and vacuole-accumulation of several hydrolytic enzymes, such as the cysteine proteases XCP1 and XCP2 (Zhao et al. 2000; Funk et al. 2002; Avci et al. 2008), the Zn^{2+}-dependent nuclease ZEN1 (Ito and Fukuda 2002) and RNases (Lehmann et al. 2001). Ca^{2+}-dependent DNases were also detected in secondary xylem cells, and their activity dynamics were closely correlated with secondary xylem development (Chen et al. 2012a). *ATMC9* encodes a caspase-like protein, which does not function as a caspase but as an arginine/lysine-specific cysteine protease (Vercammen et al. 2004). *ATMC9* was specifically expressed in differentiating vessels but not in fully-differentiated vessels (Ohashi-Ito et al. 2010). This result suggested the involvement of ATMC9 in PCD. However, application of caspase inhibitors significantly delays the time of tracheary element formation and inhibits DNA breakdown and appearance of TUNEL-positive nuclei in *Zinnia* xylogenic cell culture (Twumasi et al. 2010).

Recently, the protease responsible for developing xylem-related caspase-3-like activity was purified and identified to be 20S proteasome (Han et al. 2012). The fact that treatment with a caspase-3 inhibitor Ac-DEVD-CHO causes a defect in veins in *Arabidopsis* cotyledons, and the proteasome inhibitor clasto-lactacystin β-lactone delays tracheary element PCD in VND6-induced *Arabidopsis* xylogenic culture, strongly suggest that the proteasome is involved in PCD during this process of differentiation (Han et al. 2012). Consistent with this notion, the 26S proteasome inhibitors lactacystin and MG132 also delay or block the differentiation of suspension-cultured tracheary elements (Woffenden et al. 1998; Endo et al. 2001). Autophagy has also been suggested to be involved in tracheary element PCD (Weir et al. 2005). A recent finding that a small GTP binding protein RabG3b plays a positive role in PCD during tracheary element differentiation by activating autophagy (Kwon et al. 2010) provided support for this notion.

The PCD of xylem fibers is less well characterized compared to that of xylem tracheary elements, likely due to the fact that this process proceeds slowly in these cell types. Microarray analyses revealed that a large number of genes encoding previously-uncharacterized transcription factors, as well as genes involved in ethylene, sphingolipids, light signaling and autophagy-related factors, are expressed preferentially during xylem fiber development (Courtois-Moreau et al. 2009). Further comparison of genes related to PCD between xylem fibers and tracheary elements, in a model species like poplar, may help in advancing our understanding of PCD as it occurs in plants.

Control over master transcription factors and crosstalk between signaling pathways

It is now well established that xylem cell differentiation is regulated by various factors, both at the cell-autonomous and non-cell-autonomous level. Auxin, cytokinin, brassinosteroids and CLE peptides act, cooperatively, at different stages of xylem cell differentiation. Importantly, an as-yet-unidentified intracellular signaling system initiates the expression of genes for master transcription factors such as VND6, VND7 and SND1/NST1, each of which in turn induces distinctive xylem cell-specific gene expression. Further advances in our understanding of the events underlying xylem differentiation will be gained by studies on the related intracellular signaling pathways and the nature of the crosstalk that occurs between these specific signaling pathways.

Spatial & Temporal Regulation of Vascular Patterning

Vascular organization in leaves

The leaf vascular system is a network of interconnecting veins, or vascular strands, consisting of two main conducting tissue types: the xylem and the phloem. While the specialized conducting elements are composed of tracheary or vessel elements in the xylem and SEs in the phloem, the vascular system also contains non-conducting supporting cells, such as parenchyma, sclerenchyma and fibers. Thus, the development of cell types within the radial arrangement of the vascular bundle must be precisely spatially coordinated along with the

temporal longitudinal vein pattern in order to efficiently carry out their function as the long-distance transport system of the plant (Dengler and Kang 2001).

The spatial organization of the leaf vascular system is both species- and organ-specific. Despite the diverse vein patterns found within leaves, the one commonality that is present during the ontogeny of the vascular system is the organization of the vascular bundles into a hierarchical system. Veins are organized into distinct size classes, based on their width at the most proximal point of attachment to the parent vein (Nelson and Dengler 1997). Primary and secondary veins are considered to be major veins, not only due to their width, but because they are typically embedded in rib parenchyma, whereas higher order, or minor, veins such as tertiary and quaternary veins are embedded in mesophyll (Esau 1965a). The highest order veins, the freely ending veinlets, are the smallest in diameter and end blindly in surrounding mesophyll (**Figure 10A**).

The presence of this hierarchical system in leaves reflects the function of the veins such that larger diameter veins function in bulk transport of water and metabolites, whereas smaller diameter veins function in phloem loading (Haritatos et al. 2000). In both the juvenile and adult phase leaves of *Arabidopsis,* the vein pattern is characterized by the major secondary veins that loop in opposite pairs in a series of conspicuous arches along the length of the leaf (Hickey 1973). This looping pattern, termed brochidodromous, is present in both juvenile and adult phase leaves. However, the hierarchical pattern is well defined in the adult leaves; there is a higher vein density and vein order (up to the 6th order) when compared with the juvenile leaves (Kang and Dengler 2004). Despite this increasing vascular complexity, the overall vein pattern within a given species is highly conserved and reproducible, yet the vasculature itself is highly amenable to changes and re-modification during leaf development (Kang et al. 2007).

Longitudinal vein pattern—procambium

As indicated above, the procambium is a primary meristematic tissue that develops *de novo* from ground meristem cells to form differentiated xylem and phloem. In a temporal sense, the longitudinal vein pattern in *Arabidopsis* develops basipetally. However, the individual differentiating strands of the pre-procambium, procambium, and xylem develop in various directions (basipetally, acropetally or perpendicular to/or from the leaf midvein) depending on the stage of vascular development, as well as the local auxin levels (**Figure 10B**). Based strictly on its anatomical appearance, procambium is first identifiable by its cytoplasmically dense narrow cell shape and continuous cell files that seemingly appear either simultaneously or progressively along the length of the vascular strand (Esau 1965b; Nelson and Dengler 1997).

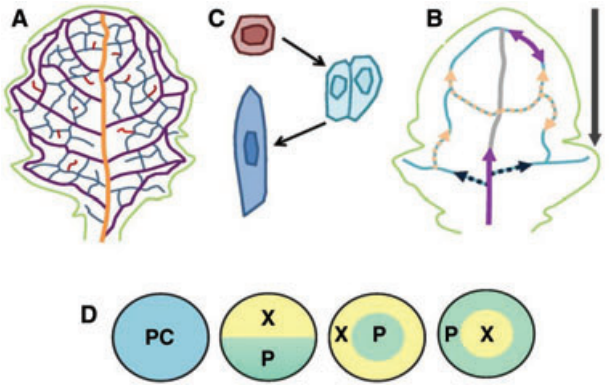

Figure 10. Longitudinal and radial vein patterning in leaves.

(A) Venation pattern in the lamina of a mature *Arabidopsis* leaf. Vein size hierarchy is based on diameter of the veins at their most proximal insertion point. Vein size classes are color coded as follows: Orange, mid (primary) vein; purple, secondary/marginal veins; blue, tertiary veins; red, quaternary/freely ending veinlets.

(B) Development of vein pattern in young leaves, as indicated by AtHB-8 (Kang and Dengler 2004; Scarpella et al. 2004). Establishment of the overall vein pattern in *Arabidopsis* is basipetal (black arrow). Secondary pre-procambium of the first pair of loops develop out from the midvein (dotted pink arrows, arrow indicates direction of pre-procambial strand progression). Pre-procambium of the second pair of secondary vein loops progresses either basipetally or acropetally. Third and higher secondary vein loop pairs progress out from the midvein towards the leaf margin and reconnect with other extending strands (dotted black arrows). Procambium differentiates simultaneously along the procambial strand (blue solid lines). Xylem differentiation occurs approximately 4 d later and can develop either continuously, or as discontinuous islands, along the vascular strand (purple lines, arrow indicates direction of xylem strand progression).

(C) Differentiation of procambial cells. Pre-procambium is isodiametric in cell shape and is anatomically indistinguishable from ground meristem cells (maroon cell). Cell divisions of the pre-procambium are parallel to the direction of growth (light blue cells) of the vascular strand, resulting in elongated shaped cells characteristic of the procambium (dark blue cell).

(D) Radial vein pattern in leaves. (Left to right): Procambial cells (as indicated by AtHB-8) are present within the vascular bundle. In a typical angiosperm leaf, xylem cells are dorsal to phloem cells (collateral vein pattern). In severely radialized leaf mutants, vein cell arrangement also becomes radialized. In adaxialized mutants such as phabulosa (phb), phavoluta (phv), and revolute (rev), xylem cells surround phloem cells (amphivasal), whereas in abaxialized mutants, such as those in the *KANADI* gene family, phloem cells surround the xylem cells (amphicribral) (Eshed et al. 2001; McConnell et al. 2001; Emery et al. 2003).

The elongated procambium cells develop through distinct cell division patterns in which they divide parallel to the vascular strand (Kang et al. 2007) (**Figure 10C**). Although the anatomical distinction of procambium is clearly evident by its elongated shape, the precursor cells, pre-procambium, are isodiametric and are anatomically indistinguishable from surrounding ground meristem cells. Due to the difficulty of clearly identifying pre-procambium and ground meristem through anatomy alone, the use of molecular markers such as *Arabidopsis thaliana HOMEOBOX 8* (*AtHB-8*), *MONOPTEROS (MP)*, and *PINFORMED 1* (*PIN1)* to identify procambium and pre-procambium has facilitated the visualization of these early stages of vascular development (Mattsson et al. 2003; Kang and Dengler 2004; Scarpella et al. 2004; Wenzel et al. 2007).

Auxin has been shown to regulate many aspects of plant development and play a critical role during vascular patterning, specifically vascular cell differentiation and vascular strand formation (Aloni 1987; Aloni et al. 2003; Berleth et al. 2000; Mattsson et al. 2003). Early classical experiments showed that auxin is capable of inducing new strands in response to wounding by promoting the transdifferentiation of parenchyma cells into continuous cell files towards the basal parts of the plant (Sachs 1981). These early observations led to the "auxin canalization hypothesis" which suggested that auxin is transported directionally through a cell, progressively narrowing into discrete canals and operating through self-reinforcing positive feedback (Sachs 1981).

In recent years, it has been shown that auxin is synthesized predominantly in leaf primordia and transported unidirectionally from apical to basal regions of the plant. Polar auxin transport (PAT) is accomplished by translocating auxin in a targeted manner through the cell, via auxin influx and efflux carriers (Lomax and Hicks 1992). Several gene families are known to affect vascular strand formation by modulating auxin levels during leaf development. The *Arabidopsis* family of efflux carriers, *PIN1*, regulates the polarity and elevated auxin levels from the shoot apical meristem into developing leaf primordia (Reinhardt et al. 2000; Benkova et al. 2003). The subcellular epidermal localization and convergence of auxin flow to the tip of the leaf primordia and subsequent basal transport of auxin directs the location of the future midvein and the sites of vascular strand formation (Reinhardt et al. 2003; Petrášek et al. 2006).

In young leaf primordia, the lateral marginal convergence points of PIN1 are required for vascular strand positioning and arrangement (Sieburth 1999; Wenzel et al. 2007). Specifically, the initial broad expression domain of auxin in the developing leaf converges and tapers to narrow cell files (presumptive vascular strands) that are dependent on auxin transport (Scarpella et al. 2006; Sawchuk et al. 2008). The large family of auxin response factors, which include transcription factors such as *MP*/*AUXIN RESPONSE FACTOR 5* (*ARF5*), plays a key role in vascular strand formation (Wenzel et al. 2007). It is now well documented that *mp* loss-of-function mutants have reduced vasculature, discontinuous veins, and also affect embryo polarity and root meristem patterning (Hardtke and Berleth 1998; Hardtke et al. 2004; Wenzel et al. 2007; Schuetz et al. 2008).

MP regulates vascular formation by inducing *PIN1* expression, and recently, MP has been shown to directly target *AtHB-8* through an activator that binds to the TGTCTG element in the *AtHB-8* promoter to induce pre-procambial expression (Donner et al. 2009). Expression of *AtHB-8* is simultaneously present along with the expression of *SHR* to demarcate presumptive vascular cells (Wenzel et al. 2007; Donner et al. 2009; Gardiner et al. 2011). Although *AtHB-8* expression is specifically localized to and remains in pre-procambial and procambial cells, the expression domain of *SHR* is localized beyond the vascular strand, suggesting an alternative function beyond vascular development in leaves (Gardiner et al. 2011).

The Class III HD-ZIP family of transcription factors, which includes *AtHB-8,* act as known regulators of both longitudinal and radial vascular patterning. The *AtHB-8* gene is one of the earliest expressed in pre-procambial and procambial strands to set up vascular patterning (Baima et al. 1995; Kang and Dengler 2004; Scarpella et al. 2004). In *Arabidopsis* leaves, longitudinal vein pattern is initiated early in development through the acquisition of pre-procambial cells along a presumptive procambial strand (Kang and Dengler 2004; Scarpella et al. 2004; Sawchuck et al. 2007). Here, *AtHB-8* is expressed in pre-procambial cells that are genetically identifiable from surrounding ground meristem cells. The distinct spatial organization of the secondary loops, characteristic of *Arabidopsis* vein patterning, develop uniquely based on the position of the secondary loop.

The pre-procambium of the first secondary loop pair develops progressively away from the point of origin, the central midvein, to form a continuous loop (**Figure 10B**) (Kang and Dengler 2004; Scarpella et al. 2004; Sawchuck et al. 2007). Later formed secondary pre-procambial strands (i.e. second or third secondary loop pairs) also develop away from the point of origin; however, the direction of the extending strand can develop either acropetally or basipetally (Kang and Dengler 2004). Procambial strands develop simultaneously along the entire length of the vascular strand (Sawchuck et al. 2007). This simultaneous occurrence of the procambial strand is commonly seen in the first secondary loop pair. Importantly, procambial strands of later formed secondary loop pairs can differentiate towards the leaf margin and reconnect with other strands (**Figure 10B**).

Approximately four d after procambium differentiation, xylem begins to develop both continuously from a point of origin or as discontinuous islands that connect either acropetally or basipetally with other strands (**Figure 10B**). To date, many

vascular pattern mutants have been identified (Scarpella and Meijer 2004; Scarpella and Helariutta 2010), and invariably, these mutants have disrupted and/or discontinuous vascular strands, suggesting that proper formation or continuity of vascular strands occurs early during the pre-procambial stages of development (Scarpella et al. 2010).

Radial vein pattern—polarity and cell proliferation

The spatial and temporal coordination of both longitudinal and radial vein pattern is essential for proper functioning of the vascular system. As leaves arise from the shoot apical meristem, the incipient leaf primordia are initially radialized, but internal tissues quickly become polarized acquiring adaxial (dorsal) and abaxial (ventral) cellular identities. The juxtaposition of adaxial and abaxial characteristics allows the leaf to grow out into a flattened lateral organ. In a typical eudicot leaf, the vascular tissues are arranged in a collateral pattern with xylem adaxial to the phloem. This differentiation must occur from a uniform procambium cell population (**Figure 10D**). Much of what we currently know concerning vascular polarity is derived from work conducted on leaf polarity mutants, as alterations in leaf polarity often result in vascular bundle defects (Scarpella and Meijer 2004; Husbands et al. 2009). In a completely radialized polarity mutant, vascular tissue is also radialized in that either xylem tissue surrounds a central cylinder of phloem (amphivasal) or phloem tissue surrounds a central cylinder of xylem (amphicribral) (**Figure 10D**).

The establishment of adaxial-abaxial polarity is temporally regulated in the shoot apical meristem. Early experiments showed that meristem-derived signals may act to promote adaxial cell fate, as leaf primordia that were altered surgically became abaxialized (Sussex 1954). However, it is unlikely that meristem-derived factors alone are sufficient in establishing organ (and vascular) polarity, and that patterning of adaxial-abaxial cell fate requires a number of genetic inputs. Transcription factors, such as those in the Class III HD-ZIP family, were identified as adaxial determinants based on radialized mutant phenotypes in *Arabidopsis*. Of these, the gain-of-function mutants in *PHB, PHV,* and *REV* display radialized leaves with amphivasal vascular bundles (McConnell et al. 2001; Emery et al. 2003). Their abaxial counterparts, such as the *KAN* genes (MYB-like GARP transcription factors) are expressed in abaxial tissues, promoting abaxial identity. Gain-of-function mutations in these genes can result in amphicribral (phloem cells outside of a ring of xylem cells) vascular bundles (Kerstetter et al. 2001; Emery et al. 2003), or in the most extreme case, result in the complete elimination of vascular tissue within the radialized organ (McConnell and Barton 1998; Sawa et al. 1999).

The intimate connection between the development of the procambium and the surrounding ground meristem (mesophyll) during tissue histogenesis has yet to be deciphered. The genetic mechanisms coupling vascular cell proliferation with organ formation during tissue histogenesis are also largely unknown. However, it is known that cell proliferation is a critical developmental process that is required during tissue histogenesis. Attaining proper cell numbers within the radial vascular bundle is essential in order for vein size hierarchy to be properly established during vascular development. The organization of this vein hierarchy is controlled, at least in part, by cell cycle regulators such as *CyclinB1;1* (Kang and Dengler 2002). Expression of *CyclinB1;1::GUS* is modulated within the vein orders so that cell cycling is prolonged in larger vein size classes, such as the midvein, but ceases first in smaller order veins. Modification of cell proliferation in leaves established that vein patterning is tightly coordinated with maintenance of meristematic competency of ground meristem cells to regulate (higher order) vein architecture (Kang et al. 2007). Although the direct association between the cell cycle and vascular patterning has yet to be determined, genes known to play a role in cell proliferation and/or stem cell maintenance may aid in elucidating this mechanistic pathway (Ji et al. 2010; Vanneste et al. 2011).

Spatio-temporal regulation of root vascular development

A number of comprehensive reviews exist that cover different aspects of root xylem development (Cano-Delgado et al. 2010; Scarpella and Helariutta 2010). In this section of the review, we will focus first on the morphological evidence for the timing of events that control vascular specification and differentiation within the root. We will then assess progress in elucidating the molecular markers and regulatory factors that govern spatio-temporal aspects of root vascular development. Spatial regulation involves cellular mechanisms that determine the arrangement of vascular cell types relative to each other.

The temporal regulation of vascular development comprises mechanisms that determine cell specification of vascular cell type lineages beyond the quiescent center (QC), as well as differences in the timing of these differentiation events (Mähönen et al. 2000). The developmental time at which various cell types differentiate can be read according to the cell's distance from the QC, or position relative to the root meristematic, elongation or maturation zone (**Figure 11A**). As the majority of research into the regulation of the spatial or temporal aspects of vascular development has been performed in the *Arabidopsis* root, we will use this model system to highlight progress in this area.

Morphological markers of root vascular development

Vasculature in the *Arabidopsis* root, as discussed earlier, is composed of the radially symmetric pericycle cell layer that surrounds the diarch vasculature. The pericycle is differentiated

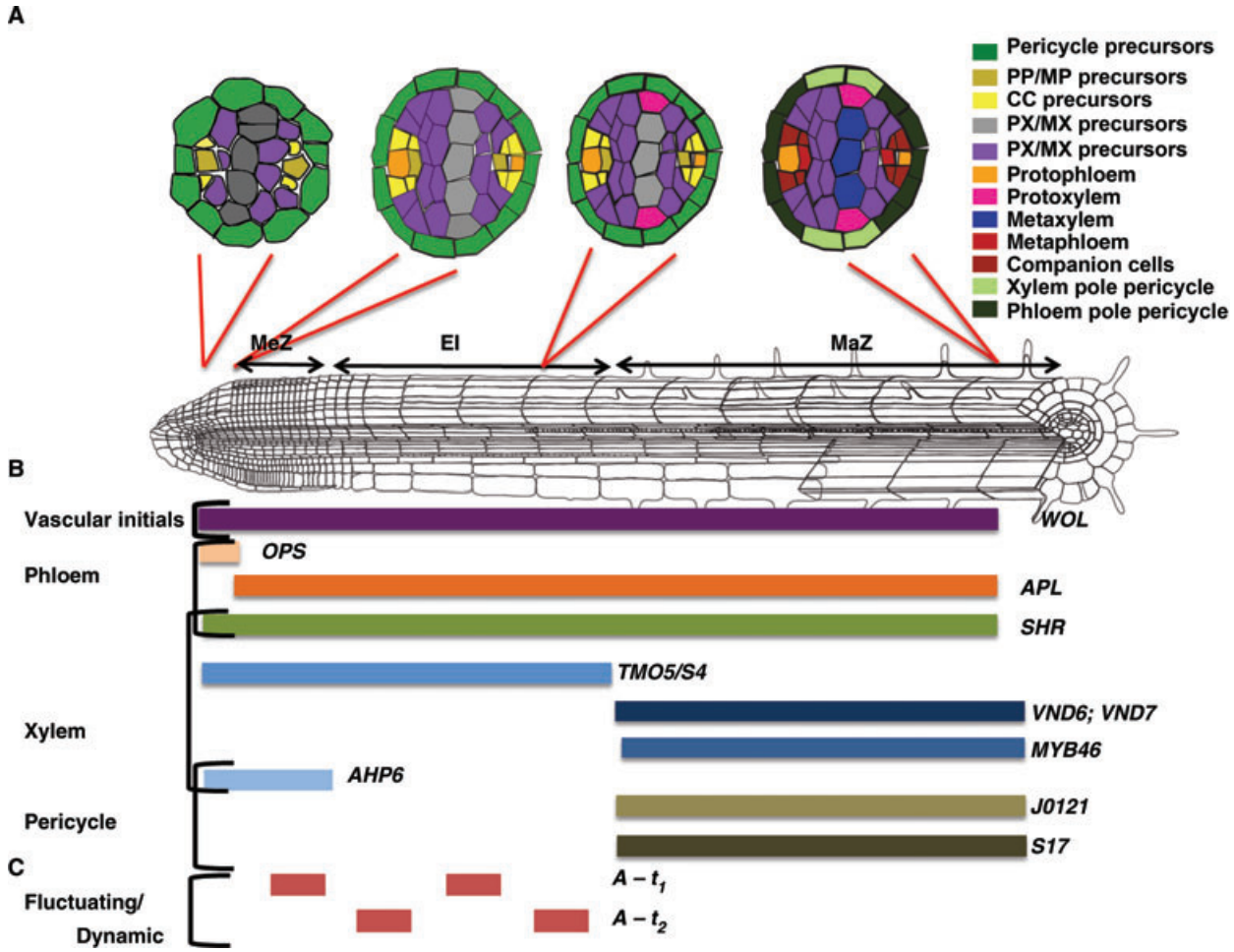

Figure 11. Spatio-temporal regulation of root vascular patterning.

(A) Spatial markers of vascular differentiation. Developmental time points at which morphological markers consistent with differentiation of vascular cell types are indicated relative to the position along the longitudinal axis of the root. Changes in differentiation are highlighted in a change in cell color. MeZ, meristematic zone; El, elongation zone; MaZ, maturation zone; PP, protophloem; MP, metaphloem; CC, companion cells; PX, protoxylem; MX, metaxylem.

(B) Temporal regulation of vascular regulator gene expression. The distinct temporal patterns of different vascular regulators are demonstrated along with the cell type with which these markers are associated. If a gene has a much higher peak of gene expression, then only this peak is shown.

(C) Examples of genes whose expression shows fluctuating peaks in developmental time (first row), or dynamic expression between roots (compare first and second rows).

into two cell types, the xylem and phloem pole pericycle cells. The former are located at the poles of the xylem axis and are the only cells competent to become lateral root primordia, whereas the latter occupy the position between the xylem poles. There are no morphological markers for phloem pole pericycle differentiation, other than their position relative to xylem pole pericycle cells, and the function of these cells remains to be elucidated.

Phloem tissue is positioned interior to the pericycle cell layer and is located at the opposing poles of the vascular cylinder, whereas the central xylem axis cells form a median line transecting the vascular cylinder, perpendicular to the two phloem poles. Procambial cells are positioned between the xylem and phloem tissues. Xylem tissue is composed of two different cell types: protoxylem and metaxylem vessels. In the *Arabidopsis* root, there are two outer protoxylem cells and three inner metaxylem cells that can be distinguished based on their secondary cell wall characteristics. Protoxylem cells have a helical or annular pattern of secondary cell wall deposition, whereas metaxylem cells have a pitted deposition pattern.

Protoxylem vessels in the root mature before the surrounding tissues elongate; during cell expansion of these surrounding cells, these protoxylem vessels are often destroyed. Thus, the metaxylem vessels act as the primary water conducting tissue throughout the main body of the plant (Esau 1965b). Metaxylem cell differentiation is temporally separated from protoxylem differentiation in that the outer metaxylem cells differentiate only after protoxylem cells differentiate and the surrounding tissues have completed their expansion. The inner metaxylem vessel differentiates later than the outer two metaxylem cells. Phloem tissue is composed of three cell types: protophloem SEs to the outside, and metaphloem SEs to the interior of the vascular cylinder, with CCs flanking the SEs. Protophloem SEs differentiate earlier than the metaphloem SEs and their associated CCs.

Detailed anatomical studies of the *Arabidopsis* root tip have elucidated the earliest events in the timing and patterning of vascular initial cell divisions that give rise to all vascular cell types in the primary root (Mähönen et al. 2000). Just above the QC, asymmetric cell divisions of vascular initial cells give rise to the presumptive pericycle layer and protoxylem cells. At a position close to the QC (~9 μm), five xylem cells are visible, and these will eventually differentiate into protoxylem and metaxylem vessels (**Figure 11A**). Two domains of vascular initials give rise to the phloem and procambial cell lineages, and they are located between 3 μm and 6 μm above the QC (Mähönen et al. 2000; Bonke et al. 2003). The number and exact pattern of future procambial cell divisions is variable between individual plants of the same species.

The full set of phloem cells (protophloem, metaphloem and CC) can be observed at a distance above the QC (~27 μm) (Mähönen et al. 2000) (**Figure 11A**). Protophloem and metaphloem SEs result from one tangential division of precursor cells, whereas CCs arise from one periclinal division of precursor cells (Bonke et al. 2003). At a further distance above the QC (~70 μm), the first histological evidence of differentiation can be observed in protophloem SEs, as determined by staining with toluidine blue (Mähönen et al. 2000). Thus, protophloem SE differentiation occurs much earlier in developmental time compared to protoxylem vessel formation (**Figure 11A**). Metaphloem SEs and CCs differentiate at an approximately similar time to the outer metaxylem SEs. However, morphological analyses have determined that the spatial patterning of xylem cells occurs temporally prior to the spatial patterning of the phloem cells within the root.

Vascular proliferation—cytokinin signaling

Vascular initial cells or stem cells are the progenitor cell type for all vascular cells within the primary root. Regulation of vascular initial cell division is the first step in vascular development and is accomplished, in part, by the two-component cytokinin receptor WOL (Mähönen et al. 2000). *WOL* is expressed early in the *Arabidopsis* embryo during the globular stage and is present throughout the vascular cylinder during all subsequent stages of embryo and primary root development (**Figure 11B**). Interestingly, vascular defects within the embryonic root have not yet been reported. In the primary root of a *wol* mutant, there are fewer vascular initial cells, and the entire vascular bundle differentiates as protoxylem. Although this suggests that *wol* is deficient in procambial, metaxylem vessel and phloem cell specification, a double mutant between *wol* and *fass* (which results in supernumerary cell layers) produces phenotypically normal procambial and phloem cells, as well as both protoxylem and metaxylem vessels. This demonstrates that the role of WOL is in vascular initial cell proliferation, and that any influence on cell specification is secondary to this defect.

Transcriptional master regulators and xylem development

Xylem cell differentiation, as marked by secondary cell wall synthesis and deposition, occurs much later in root developmental time relative to protophloem cell differentiation (**Figure 11A**). However, cells destined to become xylem cells are morphologically identifiable immediately after division of vascular initial cells. Based on gene expression data, a downstream regulator of cytokinin signaling, the AHP6, an inhibitory pseudophosphotransfer protein, is likely one of the earliest regulators of protoxylem cell specification (Mähönen et al. 2006), but is unlikely to be the sole regulator (**Figure 11B**). AHP6 functions to negatively regulate cytokinin signaling through spatial restriction of signaling within protoxylem cells. In a *wol* mutant, therefore, there is a lack of cytokinin signaling, a decrease in the asymmetric division of vascular initial cells and ectopic protoxylem cell differentiation in the few remaining vascular cells. AHP6 acts in a negative feedback loop with cytokinin signaling – cytokinin represses *AHP6* expression, while AHP6 represses and spatially restricts cytokinin signaling (Mähönen et al. 2006). Cytokinin regulates the spatial domain of *AHP6* expression in embryogenesis prior to when primary root protoxylem differentiation occurs. Thus, it appears that this negative regulatory feedback between cytokinin and AHP6 occurs upstream of protoxylem specification in the primary root (Mähönen et al. 2006).

The earliest marker of protoxylem cell specification in the primary root is achieved through a *TARGET OF MONOPTEROS 5 (TMO5)* promoter:*GFP* fusion, named S4 (Lee et al. 2006; Schlereth et al. 2010). TMO5 is required for embryonic root initiation, and expression of this bHLH transcription factor is turned on shortly after division of vascular initial cells in the primary root and is turned off prior to secondary cell wall differentiation in protoxylem cells. This marker then turns on

in metaxylem cells and subsequently turns off again prior to secondary cell wall differentiation. MYB46 is one of the transcription factors partially required for synthesis of various components of the secondary cell wall in the protoxylem and subsequent metaxylem (Lee et al. 2006; Zhong et al. 2008). This gene is expressed towards the end of the elongation zone in protoxylem cells and then later in metaxylem cells. Together, these findings suggest that, although there are no morphological markers of protoxylem cell specification early in developmental time, there are indeed molecular markers and two distinct developmental states for protoxylem and metaxylem cells: an "early" state and a "late" state. Gene expression data support this observation, as there are distinct gene expression profiles in cells marked by *TMO5* as compared to *MYB46* (Brady et al. 2007).

As mentioned earlier, PHB, PHV, REV, COR and ATHB-8 act redundantly to regulate xylem cell fate differentiation and are sufficient to regulate xylem patterning. In a dominant mutant background of PHB, *phb-1d*, which its mRNA is resistant to miRNA degradation, ectopic protoxylem is observed (Carlsbecker et al. 2010). PHB is therefore sufficient to specify protoxylem differentiation. The developmental time point at which the patterning of xylem cells is first regulated by these transcription factors remains unknown. Upstream regulators of SHR have yet to be identified, although SHR is expressed throughout the vascular cylinder during vascular development. Class III HD-ZIP transcription factors are also expressed in various cell types of the vasculature, primarily early in root developmental time in the meristematic zone; miR165a and miR166b are also expressed with a peak in endodermis cells in this same zone (Carlsbecker et al. 2010). Based solely on the temporal nature of these expression patterns, the patterning of protoxylem and metaxylem cells appears to occur prior to the action of *VND6* and *VND7*, although validation of this hypothesis requires further experimentation.

Phloem cell patterning and differentiation

Only a few factors are known to regulate phloem cell patterning and differentiation, despite protophloem being histologically evident quite early in development relative to xylem cell types (Mähönen et al. 2000). Mutations in OPS, *ops-1* and *ops-2*, result in irregular phloem differentiation within the root. In WT roots, SE and CC differentiation is first marked by cell elongation followed by callose deposition and subsequent cell wall thickening in the longitudinal dimension. In the *ops-1* mutant, cell elongation, callose deposition and cell wall thickening fail to occur within the phloem cell lineage (Truernit et al. 2012). In addition, the *ops-1* mutant has impaired long-distance phloem transport, likely because of gaps in phloem SE continuity. However, OPS is not sufficient to specify phloem cell differentiation. Overexpression of OPS results in precocious phloem cell differentiation within the root, but only within already specified protophloem and metaphloem lineages (Truernit et al. 2012).

In roots, APL is expressed later than *OPS*, that is, at a distance from the QC (Bonke et al. 2003; Truernit et al. 2012) (**Figure 11B**). In an *apl* mutant, protophloem cells are misspecified as protoxylem cells, and there is a short root phenotype (Bonke et al. 2003). Contrary to an *ops* mutant phenotype, in an *apl* mutant background, no protophloem, metaphloem, or CCs are present anywhere in the phloem pole position within the vascular cylinder. APL plays a multifaceted role in phloem development. First, APL regulates the timing of the asymmetric cell divisions that would normally give rise to the SE and CC lineages. Second, APL is required for protophloem and metaphloem SE differentiation. Third, APL represses protoxylem differentiation within cells in the phloem pole position. However, despite all these roles, APL is not sufficient to specify phloem cell specification and differentiation.

Clearly, additional factors remain to be identified in phloem cell development. The presence of a master regulator for phloem development like VND6/7 and the Class III HD-ZIP family in xylem development that is both necessary and sufficient has not been identified. Protophloem cells differentiate earlier than metaphloem cells and CCs. APL protein localization reflects this temporal difference in differentiation, but the factor(s) that determines this temporal delay has yet to be isolated. Finally, factors that determine the spatial patterning of protophloem, metaphloem and CCs are similarly unknown.

Pericycle cell specification and differentiation

Pericycle cells have been divided into two populations based on gene expression and function. Only xylem pole pericyle cells are competent to become lateral root primordia. One marker of xylem pole pericycle cell differentiation is the J0121 enhancer trap that marks xylem pole pericycle cells after they exit from the meristematic zone and pass through the elongation zone (Parizot et al. 2008). A marker of intervening cells within the pericycle tissue layer helped identify phloem pole pericycle cells. These cells are marked by expression of S17, a basic leucine zipper transcription factor. The function of phloem pole pericycle cells has not been determined, nor are there histological markers of phloem pole pericycle differentiation. However, phloem pole pericycle cells have a very distinct expression pattern and underlying transcriptional signature from that of xylem pole pericycle cells, as determined by expression profiling of marked populations of both of these cells relative to other cell types in the *Arabidopsis* root (Brady et al. 2007). Interestingly, the expression pattern of phloem pole pericycle cells more closely reflects that of cells in the developing phloem cell lineage.

No early markers of either phloem or xylem pole pericycle cells have been identified, nor have regulatory factors been

found that are both necessary and sufficient for pericycle cell specification and differentiation. Cytokinin is sufficient to suppress xylem pole pericycle differentiation, as marked by the J0121 enhancer trap line, and this, in part, requires AHP6 (Mähönen et al. 2006). MiR165/166 repression of PHB is also required for pericycle differentiation (Miyashima and Nakajima 2011). In WT roots, *AHP6* is expressed in protoxylem cells and the two abutting xylem pole pericycle cells (**Figure 11B**). In the *scr*-3 and *phb-1d* mutants, *AHP6* expression was either completely lost or detected in only one of the aforementioned three cells. In addition, expression of an additional xylem pole pericycle marker gene, *STELAR* K^+ *OUTWARD RECTIFIER (SKOR*), was greatly reduced in these *phb-1d* and *scr-3* mutant lines. Finally, in response to external auxin, which is sufficient to induce periclinal divisions in xylem pole pericyle cells, reduced periclinal divisions were observed in the *phb-1d* mutant. Expression of a mutant form of *miR165*, which is able to target the *phb-1d* miRNA-resistant *PHB* transcripts, was able to rescue the *AHP6* and *SKOR* expression patterns in *phb-1d*. However, lateral root primordium development was somewhat delayed, suggesting that SHR/miR165-dependent regulation of PHB is required for pericycle function.

Dynamic regulation of gene expression within the root vasculature

Dynamic gene expression patterns have also been identified in the root vasculature, and thus far, oscillatory expression patterns have been linked to lateral root initiation. First, oscillations in auxin responsiveness, as measured by the *DR5:GUS* synthetic auxin response reporter in xylem pole pericycle cells within the root meristematic zone, were shown to temporally correlate with lateral root initiation at regular intervals in the maturation zone (De Smet et al. 2007). Further work demonstrated that this oscillatory auxin responsiveness likely determines competence of xylem pole pericycle cells to become lateral root primordia (Moreno-Risueno et al. 2010).

To identify additional factors that play a role in lateral root development and which may act at the same time or downstream of this fluctuating auxin responsiveness, microarray analysis was used to identify genes whose expression oscillates in phase with DR5 auxin responsiveness as well as antiphase with auxin responsiveness. Many of these oscillating genes were shown to regulate prebranch initiation site formation and lateral root number (Moreno-Risueno et al. 2010). Numerous other genes have been identified that regulate dynamic expression in root developmental time. These were obtained by sectioning an individual *Arabidopsis* root into 12 successive sections, each representing a specific point in a developmental time (Brady et al. 2007). Sections of an independent root served as a biological replicate.

Based on these data, genes were identified whose expression fluctuates over root developmental time; e.g., they showed peaks of expression in the meristematic zone and maturation zone, with downregulation of expression in the meristematic zone (**Figure 11C**). A rigorous statistical method was developed to identify cases of dynamic expression between roots (**Figure 11C**), and many of these were expressed specifically in root phloem cell types or in xylem pole pericycle cells. Their function was inferred to be associated with energy capture and lateral root initiation, respectively (Orlando et al. 2010). Together, these data indicate that oscillatory, rhythmic and fluctuating gene expression within roots and between roots in the root vasculature serve to regulate patterning of vascular cells, and likely other vascular biological functions.

Spatio-temporal regulation of root and shoot vascular development and connectivity

Studies on *Arabidopsis* root mutants defective in cell proliferation and cell differentiation may provide insight into possible common genetic regulatory mechanisms controlling radial vascular development in shoots and leaves. Mutants such as *wol* show decreased cell proliferation in root procambium and differentiate completely into protoxylem (Mähönen et al. 2000), whereas the *apl* mutant shows defects in phloem development (Bonke et al. 2003). Although these genes appear to affect root cells exclusively, recent studies revealed that *SHR* is also involved in regulating cell proliferation in leaves (Dhondt et al. 2010). Furthermore, expression of *SHR* is tightly correlated with *AtHB-8* expression during vascular strand formation (Gardiner et al. 2011). While the spatial and temporal patterns of cell proliferation during root vascular development are becoming clearer, understanding this mechanism in leaves has proved to be more challenging. It is likely that combinatorial control of both hormones and genetics are in effect, and that these components are tightly integrated in both tissue and organ (leaf) morphogenesis. Therefore, isolating these developmental components will be critical in order to thoroughly understand the spatial and temporal control of vascular development in leaves.

Secondary Vascular Development

The term “secondary growth” refers to the radial growth of stems, and is ultimately the result of cell division within a lateral meristem, the vascular cambium (Larson 1994). The vascular cambium produces daughter cells towards the center of the stem which become part of the secondary xylem, or wood (**Figure 12**). The cambium also produces daughter cells towards the outside of the stem which become part of the inner bark. There are two types of cambial initials: fusiform and ray.

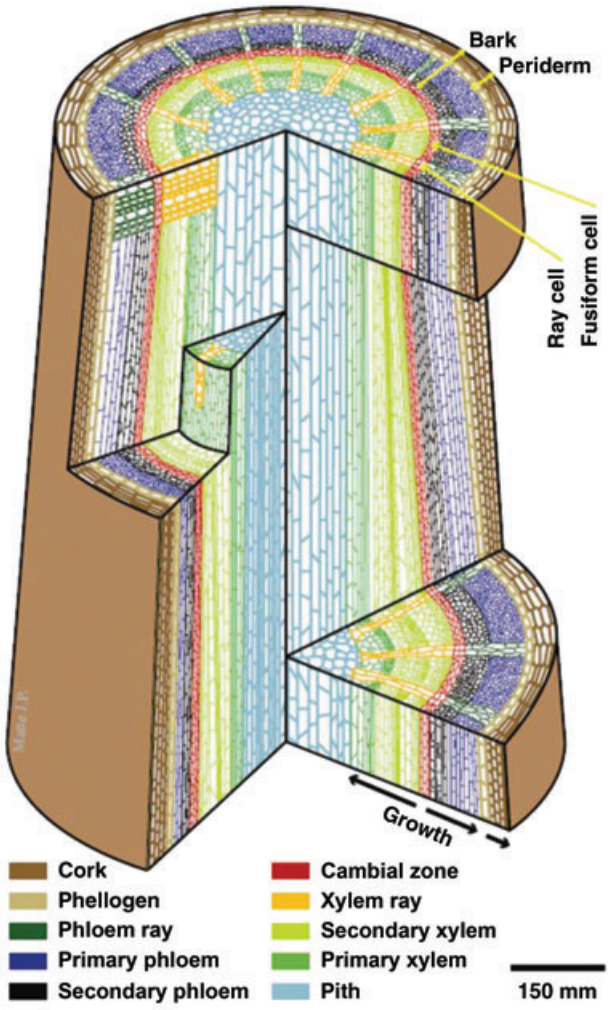

Figure 12. Internal structure of a woody plant stem.

The vascular cambium consists of a centrifugal layer of fusiform secondary phloem and a centripetal layer of secondary xylem cells surrounding a central zone comprising phloem and xylem transit amplifying cells with a central uniseriate layer of cambial stem cells. Most angiosperms and gymnosperm trees species also contain radial files of near isodiametric ray cells that play a role in nutrient transport and storage (reproduced from Matte Risopatron et al. (2010) with permission).

Fusiform initials give rise to the vertically-oriented cells, including water-conducting tracheary elements of the secondary xylem, and nutrient- and molecular signaling-conducting SEs of the secondary phloem. Ray initials produce procumbent cells that serve to transport materials radially in the stem, and likely serve storage and other functions that are currently poorly defined.

To produce a functional, woody stem, these and many other developmental processes must be coordinated (Du and Groover 2010). Importantly, these developmental processes are also highly influenced by environmental cues. This is evident when observing annual rings in a tree stump, where favorable environmental conditions in the spring can lead to rapid growth and production of wood with anatomical and chemical differences from wood produced under draught and less favorable conditions later in the growing season. Another notable example of how environmental cues influence secondary growth is the formation of reaction wood in response to gravity and mechanical stresses, with reaction wood serving to right bent stems or to support horizontal branches (Du and Yamamoto 2007).

In this section of the review, we will highlight some of the more recent advances in the understanding of how secondary growth is regulated. This is an exciting period in the study of secondary growth, as genomic approaches applied to a number of species have yielded comprehensive lists of the genes expressed in the cambium and secondary vascular tissues. Additionally, the model forest tree genus *Populus* now has a fully sequenced genome (Tuskan et al. 2006), which, when paired with relatively efficient transformation systems for some *Populus* genotypes, has allowed detailed functional characterization of a modest number of regulatory genes.

One emerging theme from these studies is that at least some of the major regulatory genes and mechanisms that regulate the cambium and secondary vascular development have been either directly co-opted from the shoot apical meristem, or else represent genes derived from duplication of an ancestral shoot apical meristem regulator (Spicer and Groover 2010). Thus, the study of the cambium and secondary growth also presents opportunities to understand the evolution of meristems and details as to how regulatory modules of genes can be reused and repurposed during plant evolution. Furthermore, since secondary growth in angiosperms, and perhaps in both angiosperms and gymnosperms, is likely homologous, advances in our understanding within model species like *Populus* can potentially greatly accelerate our understanding of secondary growth in less tractable species.

The many values of secondary woody growth

To better understand the importance of the fundamental processes involved in secondary growth, it is worthwhile to first gain an understanding of the practical reasons of how research of secondary growth is important to ecosystems, societies and industries. The wood produced by forest trees during secondary growth represents durable sequestration of the greenhouse gas CO_2, which is ultimately incorporated into wood as byproducts of photosynthesis. Wood is primarily composed of fiber and tracheary element cells which undergo complex processes of differentiation in which they synthesize thickened secondary cell walls before undergoing PCD to produce cell corpses.

Fibers impart mechanical strength to wood, whereas tracheary elements provide both mechanical strength as well as water conduction. Lignin and cellulose are primary components of secondary cell walls, and thus of wood, and impart mechanical strength and resistance to degradation. Together, cellulose and lignin are the most abundant biopolymers on the planet.

The secondary cell walls in wood ultimately reflect storage of energy and CO_2 derived from photosynthesis. With regards to carbon sequestration, forests are second only to the oceans in the biological sequestration of carbon, and are thus central to the carbon cycle and to mediating the levels of atmospheric CO_2. The energy stored in wood has played central roles in history by providing heating and cooking fuels, and continues to play these vital roles in developing countries today (Salim and Ullsten 1999; FAO 2008). Looking to the future, the woody tissues of trees are increasingly of interest as a net-carbon neutral source of bioenergy (FAO 2008). While wood wastes have long played roles in cogeneration plants to supplement coal or to provide energy to forest industry mills, more recently woody biomass is being utilized as a "next generation" biofuel. Wood from trees can be utilized as a feedstock to produce ethanol, syngas, or other biofuels. In addition, similar to a petroleum refinery, the utility and economics of biorefineries will be bolstered by production of value added products such as acetic acid and other chemicals (Naik et al. 2010). Fast growing woody perennial crops, including forest trees such *Populus*, are beginning to be utilized as a source of biofuels on industrial scales (Sannigrahi et al. 2010).

Wood is also used to produce pulp, paper, lumber, and countless derived forest products. Forest products have been estimated to represent three percent of the total world trade (FAO 2009). Harvesting and processing of wood products, as well as related follow-on industries, represent vital components of rural economies in forested regions worldwide, where few alternative industries exist. Importantly, forests and the woody bodies of trees provide numerous ecosystem services to which it is difficult to ascribe an economic value (Salim and Ullsten 1999; FAO 2009). Forests provide unique habitats, underpin crucial ecosystems, provide clean water, and are the focus of tourism industries worldwide. Perhaps the most difficult to value are the aesthetic, cultural, and spiritual aspects of forests, trees, and wood. In short, wood produced by forests is central to the health of our planet and society.

The biology and regulation of secondary growth

Secondary growth represents the culmination of a number of fascinating developmental processes. Currently, there are mechanisms identified and partially characterized that regulate specific developmental processes, including cambium initiation and maintenance, tissue patterning, and the balance of cell division and cell differentiation. While our understanding of secondary growth is far from complete, past and ongoing genomic studies have provided exhaustive lists of all the genes expressed during secondary growth. Molecular genetics studies have provided insights into the function of a modest number of regulatory genes, primarily encoding transcription factors, signaling peptides, and receptors. Plant growth regulators, including cytokinin, ethylene, gibberellic acid and most notably auxin, have all been implicated in influencing some aspect of secondary growth.

Here, we provide a brief analysis of some of the mechanisms identified that regulate secondary growth. First, we will focus on auxin, as it has profound influences on secondary growth. Auxin has long been known to be a critical regulator of cambium functions and secondary growth. For example, exogenous auxin applied to decapitated shoots can stimulate cambial formation and activity (Snow 1935). It is generally assumed that auxin is produced in leaves and apical meristems, and transported down the cambium to stimulate growth. However, direct determinations of the actual routes of auxin transport in the stem and the relative amount of auxin synthesized in the stem are currently lacking. Indeed, looking at trees like the giant sequoia in which the canopy foliage can be a hundred feet from the active cambium at the base of the stem, bring into question models for auxin synthesis and transport that were developed in smaller and more tractable plant species.

The role of auxin transport and auxin gradients during secondary growth have been researched directly in forest trees (Uggla et al. 1996; Schrader et al. 2003; Kramer et al. 2008; Nilsson et al. 2008), but remain inconclusive. A radial auxin gradient is present across secondary vascular tissues and peaks in the region of the cambium and nascent secondary xylem in both angiosperm and gymnosperm trees (Uggla et al. 1996; Tuominen et al. 1997). This observation spurred speculation that auxin could create a radial morphogen gradient, but that concept has been brought into question by studies showing that few genes that are auxin-responsive actually show peaks of expression in the cambial zone (Nilsson et al. 2008).

Auxin has also been shown to be transported basipitally in the stem, and to be involved with polarity determination in secondary vascular tissues (Kramer et al. 2008). Genes encoding PIN-type auxin efflux carriers are preferentially expressed in the cambial zone and developing xylem in the radial gradient, and in the apical-basal gradient they show a sharp peak of expression in internodes that are transitioning to secondary growth (Schrader et al. 2003). Currently, the subcellular localization and function of PIN transporters is largely uncharacterized in stems, and the functional significance of the auxin gradients remains contentious (Nilsson et al. 2008). Thus, auxin is a central point for important new advances in the study of secondary growth.

Indirect observations suggest that cell-cell communication might play important roles in secondary growth, and studies

are beginning to reveal important mechanisms in how the balance of cell differentiation and cell division is regulated, and how tissue identity is maintained across layers of secondary vascular tissues. Recent studies in *Arabidopsis* and *Populus* have identified mechanisms by which the balance of cell differentiation and tissue identity are established through cell-cell signaling. As previously discussed, in *Arabidopsis*, TDIF is a small peptide product of the phloem-expressed *CLE41/44* gene (Ito et al. 2006). TDIF is secreted from the phloem, and acts to inhibit tracheary element differentiation and stimulate cambial activity (Ito et al. 2006; Hirakawa et al. 2008; Etchells and Turner 2010), as well as to regulate the orientation of cambial divisions (Etchells and Turner 2010). This peptide is perceived by the LRR-Receptor kinase Phloem Intercalated with Xylem (PXY), which is expressed in the procambium (Hirakawa et al. 2008; Hirakawa et al. 2010). Loss-of-function *pxy* mutants show phloem cells intermixed in the xylem (Fisher and Turner 2007). TRIF/PXY signaling activates *WOX4* (Hirakawa et al. 2010), which encodes a transcription factor that presumably influences gene expression associated with meristematic cell fate. Interestingly, stimulation of cambial activity by auxin requires functional WOX4 and PXY (Suer et al. 2011), providing insight into how auxin integrates into transcriptional regulation in secondary vascular tissues.

Patterning and polarity in secondary vascular tissues

Cross sections of a typical woody stem show that secondary vascular tissues are highly patterned (**Figure 12**), and that the proper position and patterning of the cambium, secondary xylem, and secondary phloem are crucial to the function of these tissues. Additionally, secondary vascular tissues can be described in terms of polarity, analogous to vasculature in leaves. Take for example the vasculature of a typical dicot tree, poplar, in which the vascular bundles in leaves always have xylem towards the adaxial and phloem towards the abaxial surface of the leaf. By following those vascular bundles through the leaf trace and into the stem, it becomes apparent that the same polarity relationships are found in both primary and secondary vascular tissues, with xylem towards the center and phloem towards the outside of the stem.

Insights into how polarity is established and maintained in vascular tissues has been provided by pioneering studies of the Class III HD-ZIP and KANADI transcription factors in *Arabidopsis*. These Class III HD-ZIPs are highly conserved in plants, act antagonistically with KANADIs, and have been shown to regulate fundamental aspects of meristem function, polarity, and vascular development (Emery et al. 2003; Izhaki and Bowman 2007; Bowman and Floyd 2008). In *Arabidopsis*, *REV* is implicated in various developmental processes, including patterning of primary vascular bundles (Emery et al. 2003; Bowman and Floyd 2008). A recent study showed that misexpression of a *Populus REV* ortholog results in formation of ectopic cambia in the cortex of the stem, and that these cambia can produce secondary xylem with reversed polarity (Robischon et al. 2011), indicating that the Class III HD-ZIPs also affect patterning and polarity in secondary vasculature.

Genetics and genomics: critical tools for advancing knowledge on woody growth

Research on secondary growth is at an exciting point, as genomic tools are now allowing the characterization of the genetic variation within species that is responsible for wood quality and growth traits. Association mapping is being taken to a whole-genome scale for some tree species, and will undoubtedly provide fascinating insights into macro- and micro-evolution of wood formation (Neale and Kremer 2011). Genomic tools are also now enabling the first generation of network biology approaches in the model tree genus *Populus* (Street et al. 2011) that can be used in understanding woody growth. Such approaches utilize a variety of genomic data types to model the genetic networks that regulate specific aspects of woody growth, and can ultimately produce a “wiring diagram” of regulatory networks. This will be important for both better directing future research and for providing predictive models that can potentially be used to better direct breeding programs, identify regulatory genes for biotechnology, and provide insights into the complexities of biological processes fundamental to the future of forests worldwide.

Physical and Physiological Constraints on Phloem Transport Function

We now turn our attention to an examination of the constraints of photoassimilate transport in the most evolutionarily advanced plants, the angiosperms. Here, photoassimilate conducting units are comprised of SEs arranged end-to-end to form conduits that are referred to as sieve tubes. At maturity, SEs lack nuclei and vacuoles, and their parietal cytoplasm has a greatly reduced number of organelles. In contrast to xylem tracheary elements, SEs retain their semi-permeable plasma membrane. Their shared end walls contain interconnecting pores (sieve plate pores) formed from PD coalescing within pit fields (Evert 2006). In addition, each SE is highly interconnected symplasmically with a metabolically active CC, through specialized PD, to form a functional unit referred to as the SE-CC complex.

In order to provide a framework on which to identify the physical and physiological constraints regulating phloem transport, we must first examine the physical mechanisms responsible for resource transport through the sieve tube system. As photoassimilate flow is polarized from source leaves (net exporters of resources) to heterotrophic sinks (net importers

of resources), phloem transport can be envisaged in terms of three key physiological components arranged in series. These components are: (a) loading of photosynthate (most commonly sucrose) in collection phloem (minor veins) within source leaves, (b) long-distance delivery in transport phloem, and (c) unloading from release phloem (**Figure 13A**). Principles of flux control analysis dictate that each component will confer an influence (constraint) on overall flow, from source to sink; thus, the question of constraint becomes one of degree.

Bulk flow identifies the regulatory elements

The phloem is generally buried deep within plant tissues, and this location, along with its sensitivity to mechanical perturbation, has made it technically challenging to observe flows through sieve tubes, non-invasively and in real-time. However, there is a critical body of experimental evidence showing that solutes and water move at similar velocities through sieve tubes (van Bel and Hafke 2005; Windt et al. 2006). These studies suggest that the phloem translocation stream moves by bulk flow, consistent with the now widely accepted pressure flow hypothesis put forward originally by Münch (1930).

On the premise that transport through sieve tubes conforms to bulk flow, then the transport rate (R_f) of a nutrient species is given by the product of transport velocity (V), sieve tube cross-sectional area (A) and phloem sap concentration (C), whereby:

$$R_f = V \cdot A \cdot C \quad (1)$$

Both A and C are finite elements, whereas, given that flow through sieve tubes approximates laminar flow through capillaries, the factors determining transport velocity (V), or solvent volume flux (J_v, having units of $m^3\ m^{-2}\ s^{-1}$, or $m\ s^{-1}$), are identified by the Hagen-Poiseuille Law as:

$$J_v = \Delta P \pi r^2 / 8 \eta l \quad (2)$$

Here, ΔP is the hydrostatic pressure difference between either end of each sieve tube of length (l), the translocation stream has a viscosity (η), and it moves though sieve tubes of known radii (r) that have sub-structural elements that serve to impede transport; i.e., sieve plate pores and parietal cytoplasm (Mullendore et al. 2010).

The water potential (ψ_w) of any cell (e.g., a SE-CC complex) is given by:

$$\psi_w = \psi_P + \psi_\pi \quad (3)$$

where ψ_P is the pressure potential and ψ_π is the osmotic or solute potential within the cell of interest. The value of ψ_P within a cell is determined by the magnitude of ψ_π and the ψ_w of the cell wall (apoplasmic potential). As ψ_π in the wall is generally close to zero, and given that the cell is in quasi water potential equilibrium with its wall (i.e., the values of ψ_w for the cell and its surrounding wall [apoplasm] are close to being equal), then the value of ψ_P in the cell (CC-SE) is given by:

$$\psi_P = \psi_w - \psi_\pi \quad (4)$$

Armed with the above information, we can identify key elements that may constrain rates of phloem transport.

Sieve element osmotic potentials are determined by phloem loading/retrieval

The ψ_π of the SE content (generally termed phloem sap) is primarily (60% to 75%) determined by one of several sugar species (sucrose, polyol or a raffinose family oligosaccharides – RFOs) with K^+ and the accompanying anions accounting for most of the remaining osmotic potential (Turgeon and Wolf 2009). Thus, loading/retrieval of sugars plays a key role in setting the SE hydrostatic pressure (Equation 4), and, for this reason, we shall primarily focus attention on sugar transport, but where appropriate, we will comment on the involvement of other solutes.

Proposed phloem-loading mechanisms are based on thermodynamic considerations and cellular pathways of loading. The most intensively studied is the apoplasmic loading mechanism in which sucrose (or polyol) loading requires the direct input of metabolic energy (**Figure 13B**, **I**), and is widespread amongst monocot and herbaceous eudicots species. An energy-dependent symplasmic loading mechanism also has been described. Here, sugar (sucrose or polyol) diffuses down its concentration gradient through a symplasmic route from mesophyll cells to specialized CCs, termed intermediary cells (ICs), where biochemical energy is required for sucrose/polyol conversion to large RFOs. This loading mechanism is referred to as the polymer trap mechanism (**Figure 13B**, **II**) and is thought to operate predominantly in herbaceous eudicots.

Another loading system has been suggested to operate in woody plants (Davidson et al. 2011; Liesche and Schulz 2012) in which sugars are passively loaded through diffusion, driven by high sugar concentrations maintained in the mesophyll cell cytosol (Rennie and Turgeon 2009; Turgeon 2010a). This pathway is considered to be symplasmic, based on observed high PD densities between each cellular interface from mesophyll to SE-CC complexes (**Figure 13B**, **III**). It has also been suggested that delivery of sugars into SE-CC complexes could be achieved by bulk flow operating through interconnecting PD (Voitsekhovskaja et al. 2006).

Several caveats must be considered for both the symplasmic diffusion and bulk flow models for passive phloem loading. If PD were to allow diffusion of sugars from mesophyll cells into sieve tubes, then all similarly-sized metabolites and ions should also pass into SE-CC complexes; i.e., the system would lack specificity. For situations in which bulk flow might transport sucrose, again all other soluble constituents present within the cytosol of cells forming the loading pathway should also gain

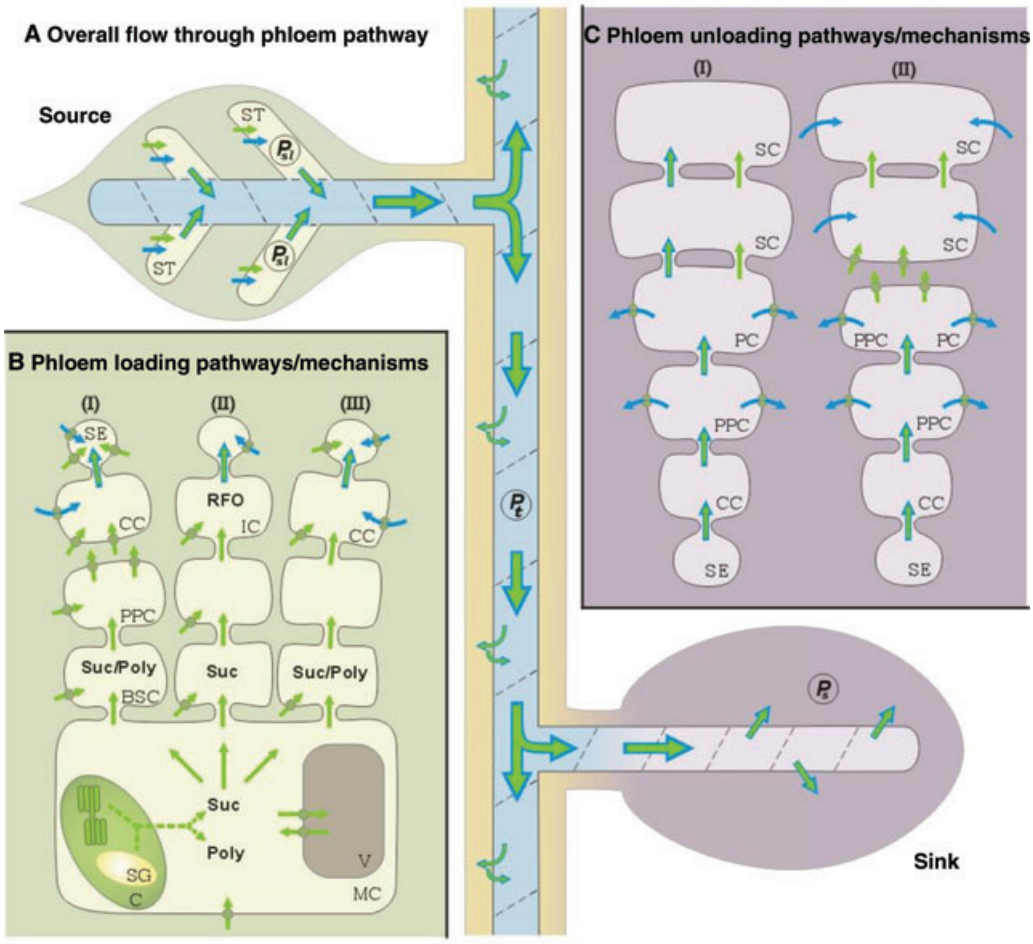

Figure 13. Diagrammatic representation of resource flow through the phloem pathway.

(A) Overall flow of resources through the phloem pathway. Within source leaves, resources (nutrients – green arrows and water – blue arrows) are loaded into sieve tubes (ST) of the collection phloem (chalk) formed by a network of minor veins. These loaded solutes lower ψ_π in the ST that then causes water to move, by osmosis, across the ST semi-permeable plasma membrane resulting in a high ψ_{Psl} condition. This osmotically-driven increase in ψ_P serves as the thermodynamic driving force to drive bulk flow (green highlighted blue arrows) of ST sap throughout the phloem system. In this context, loaded resources flow from collection phloem STs into STs of lower order veins, functioning as transport phloem (light blue) for export from source leaves. Transport phloem supports long-distance axial transport of resources from source leaves to sinks through STs of exceptionally high hydraulic conductivities that homeostatically sustain their ψ_P by resource exchange with surrounding tissues (curved green superimposed on blue arrows). Upon reaching the release phloem (light mauve), resources are unloaded from STs by bulk flow through plasmodesmata (PD) that interconnect the surrounding cells. The difference in pressure potential between the source and sink ($\psi_{Psl} - \psi_{Ps}$) represent the hydrostatic pressure differential that drives bulk flow through the phloem pathway from source to sink.

(B) Phloem loading pathways and mechanisms within source leaves. Photosynthetically reduced carbon, generated in chloroplasts, is used to drive sucrose (Suc) or polyol (Poly) biosynthesis (green broken arrows) within the cytosol of mesophyll cells (MC). Excess Suc/Poly is transiently stored in vacuoles (V) of mesophyl cells and, along with carbon from remobilized chloroplastic starch grains (SG), buffers their cytosolic pool sizes available for phloem loading. Suc/poly (green arrows) moves from mesophyll cells along a phloem-loading pathway that includes bundle sheath cells (BSC), phloem parenchyma cells (PPC), companion cells (CC) or intermediary cells (IC) to finally enter sieve elements (SE) of the collection phloem. Three loading mechanisms are considered to function in different species. (I) Active apoplasmic loading: Suc (and/or Poly) is first released from phloem parenchyma cells (PPCs) by the action of a permease (Chen et al. 2012b), and subsequently retrieved into SE-CC complexes by symporters located along SE-CC plasma membranes. (II) Symplasmic loading: Raffinose oligosaccharides (RFOs) are synthesised in specialized CCs, termed ICs, from Suc delivered symplasmically from MCs. The larger molecular

entry into the phloem. When operating over a prolonged period, either of these proposed passive-loading systems would be anticipated to cause a perturbation to metabolism within the mesophyll cells.

Rates of phloem loading depend upon the pool size of each transported solute available for loading, as well as the loading and retrieval mechanisms. For sugars, sucrose (Grodzinski et al. 1998) and polyol (Teo et al. 2006) pools are generated in mesophyll cells, whereas RFOs are synthesized from sucrose that enters the specialized ICs (Turgeon and Wolf 2009) (**Figure 13B**, **II**). Irrespective of sugar species and phloem loading mechanism, during the photoperiod, transported sugars arise from current photosynthesis and export rates are linked positively with the sugar pool size (Grodzinski et al. 1998; Leonardos et al. 2006; Lundmark et al. 2006). During the night, sugar pools are fed by starch reserves remobilized from chloroplasts (Smith and Stitt 2007) and sugars released from vacuolar storage in mesophyll cells (Eom et al. 2011) (**Figure 13B**). Depending upon carbon gain by leaf storage pools during the preceding photoperiod, remobilizing reserves during the night can sustain sugar pool sizes and, hence, export rates (Grimmer and Komor 1999).

In situations where source leaves are operating at suboptimal photosynthetic activity, analyses of metabolic control have provided estimates that source leaf metabolism exercised approximately 80% of the control exerted over photoassimilates transported into developing potato tubers (Sweetlove et al. 1998). However, the relationship between leaf metabolism and export rates also depends upon prevailing source/sink ratios. This can be illustrated by studies aimed at investigating effects associated with CO_2 enrichment. Under conditions of source limitation, leaf photosynthetic rates are increased substantially by CO_2 enrichment, and are matched proportionately by those of photoassimilate export (Farrar and Jones 2000). In contrast, more attenuated responses of leaf photosynthetic rates are elicited by CO_2 enrichment under sink limitation, and these are not proportionately matched by export (Grodzinski et al. 1998; Grimmer and Komor 1999). The latter response suggests that, under sink limitation, predominant control of photoassimilate transport shifts to processes downstream of source leaf sugar metabolism.

Estimates of membrane fluxes of sucrose loaded into SE-CC complexes in sugar beet leaves fall into the maximal range for plasma membrane transporter activity (Giaquinta 1983). Therefore, if sucrose transporters are indeed operating at maximum capacity, then their overexpression might be expected to result in enhanced rates of phloem loading and photoassimilate export. However, overexpression of the spinach sucrose transporter (SoSUT1) in potato, while altering leaf metabolism, exerted no impact on biomass gain by the tubers (Leggewie et al. 2003). This finding indicates an absence of any constraint imposed by endogenous sucrose transporters on phloem loading. Indeed, phloem loading can respond quite rapidly (within minutes) to changes in sink demand (Lalonde et al. 2003).

A striking example of the dynamic range available to the phloem loading system is shown by studies performed on *Ricinus,* a plant whose phloem sap will exude (bleed) from severed SE-CC complexes. Here, excisions made in *Ricinus* stems reduced ψ_P to zero in this region of the sieve tube system. This treatment resulted in exudation of phloem sap from the severed sieve tubes and an increase in translocation and, hence, phloem-loading rates of sucrose, by an order of magnitude (Smith and Milburn 1980a). This observed rapid response is envisaged to reflect signaling from the sink region (in this case, the site of SE-CC stem excision) to source leaves. Here, pressure-concentration waves transmitted through interconnecting sieve tubes (Mencuccini and Hölttä 2010) could act to regulate transporter activity mediating phloem loading (Smith and Milburn 1980b; Ransom-Hodgkins et al. 2003).

←

sizes of RFOs are thought to prevent their backward diffusion through PD interconnecting ICs with PPCs; however, the dilated PD that interconnect the IC-SE complexes permit forward diffusion of RFOs into the SEs (polymer trap model). (III) Passive – symplasmic loading: Suc or Poly is proposed to move by diffusion through the symplasm, via PD, down their concentration gradients from MCs to SEs. For all phloem-loading mechanisms, water enters (curved blue arrows) SE/CC or IC complexes through aquaporins (paired khaki ovals) (Fraysse et al. 2005).

(C) Phloem unloading pathways from release phloem in sink organs. In all sinks, it is highly probable that imported resources are unloaded symplasmically by bulk flow from release phloem SE-CC complexes (green-highlighted blue arrows) into adjacent PPCs. Onward resource movement through the phloem-unloading pathway may occur by the following pathways. (I) Continuous symplasmic unloading: here, resources (green-highlighted blue arrows) likely continue to move by diffusion through PD into surrounding phloem parenchyma (PC) and ultimately sink cells (SC). (II) Apoplasmic step unloading: here, the phloem-unloading route involves resource transit through the sink apoplasm due to a symplasmic discontinuity in unloading pathways at either the PPC/SC or PC/SC interface. Membrane exchange of nutrients to, and from, the sink apoplasm occurs by transporter-mediated (khaki circles) membrane efflux and influx mechanisms, respectively. In both cases, water exiting SE-CC complexes can enter SCs (in the case of growing sinks) or, for non-expanding storage sinks, water returns to the xylem transpiration stream by exiting PPC/PCs (blue curved arrows) to the sink apoplasm through aquaporins (paired khaki ovals).

Other layers of post-translational control of sucrose transporter activity include protein-protein interactions, e.g., SUT1-SUT4 regulation of phloem loading (Chincinska et al. 2008), redox-induced dimerization of SUT1 (Krügel et al. 2008), and cytochrome b5 interaction with MdSUT1 and MdSOT6 (Fan et al. 2009). Interestingly, the question as to whether symplasmic loading species also have the capacity for short-term adjustments in phloem loading capacity is less certain (Amiard et al. 2005).

Magnetic resonance imaging studies, conducted on long-distance transport of water through the vascular system in a range of species, have established that phloem transport remains unaffected by diurnal variations in transpiration-driven changes in apoplasmic leaf water potential (Windt et al. 2006). Thus, a regulatory mechanism must operate to maintain a constant pressure gradient ($\Delta\psi_P$) to drive bulk flow through sieve tubes. This likely involves osmoregulatory activities at the level of the SE-CC complex (Pommerrenig et al. 2007).

In general, it would appear that phloem loading of major osmotic species (sugars and K^+) does not constrain phloem transport under optimal growth conditions. During periods of abiotic stress, phloem loading can minimize the impact of water/salt stress through osmoregulatory activities of cells comprising the phloem-loading pathway(s) (Koroleva et al. 2002; Pommerrenig et al. 2007). Interestingly, and perhaps surprisingly, both apoplasmic (Wardlaw and Bagnall 1981) and symplasmic (Hoffman-Thom et al. 2001) loaders undergo maintenance of phloem loading activities in cold-adapted plants. This indicates that changes in the viscosity of the phloem translocation stream may have little impact on bulk flow through sieve tubes. In contrast, elevated temperatures can slow translocation by callose occlusion of sieve pores (Milburn and Kallarackal 1989). In addition, deficiencies of K^+ and Mg^{2+} can impact apoplasmic loading of sucrose into SE-CC complexes (Hermans et al. 2006). In the case of K^+, this is thought to reflect a limitation in charge compensation across the SE-CC plasma membrane which could impede the operation of the sucrose-H^+ symport system (Deeken et al. 2002), whereas Mg^{2+} deficiency could lower the availability of Mg^{2+}-ATP which serves as substrate for the H^+-ATPase that generates the proton motive force to power the sucrose H^+ symporter (Cakmak and Kirkby 2008).

In terms of minor osmotic species, direct control of their phloem translocation rates is determined entirely by the concentrations to which they accumulate in SE-CC complexes. This situation is nicely illustrated by studies performed on transgenic peas expressing a yeast *S*-methylmethione transporter under the control of the phloem-specific *AtAAP1* promoter. Here, *S*-methylmethione levels in developing seeds were found to be proportional to their concentrations detected in phloem exudates (Tan et al. 2010).

Mechanisms of phloem unloading

The cellular pathway of phloem unloading may extend, functionally, from SE lumens of the release phloem to sites of nutrient utilization/storage in the particular sink organ/tissue (Lalonde et al. 2003; **Figure 13C**). Within these bounds, the cellular pathways followed circumscribe the physical conditions under which an unloading mechanism operates. Most sink systems investigated to date have PD interconnecting the SE-CC complex to cells of the surrounding ground tissues, and, thus, confer the potential for universal symplasmic unloading (**Figure 13C**, **I**). In general, such routes for symplasmic unloading have low densities of PD that interconnect SE-CCs with adjacent phloem parenchyma cells. Thus, a marked bottleneck for symplasmic nutrient delivery may exist at this cellular interface.

Unloading routes in a variety of sink systems have been mapped by using membrane-impermeant fluorochromes loaded into phloem of source leaves. Upon import into the release phloem zone, fluorochrome movement can be retained within the vascular system of fleshy fruit during their major phase of sugar accumulation (e.g., apple, a sorbitol transporter (Zhang et al. 2004), grape berry, a sucrose transporter (Zhang et al. 2006), and cucumber, an RFO transporter (Hu et al. 2011). However, more commonly, the fluorochrome moves symplasmically out from the phloem into surrounding ground tissues (**Figure 13C**, **I**) as found for root and shoot meristems (Stadler et al. 2005), expanding leaves (Stadler et al. 2005), young fruit prior to their major phase of sugar accumulation (Zhang et al. 2006), and developing seeds in which movement is restricted to maternal tissues (Zhang et al. 2007).

In developing fruits, during the phase of sugar accumulation, SE-CC complexes are thought to be the site of sucrose release into the fruit apoplasm (Zhang et al. 2004, 2006; Hu et al. 2011). Studies conducted on tomato fruit have indicated that the cumulative membrane surface area of SE-CC complexes would be barely adequate to support sucrose unloading at maximal fluxes known to be associated with membrane transport. In contrast, using the range of reported PD-associated fluxes (Fisher 2000), it can be shown that PD densities could readily accommodate unloading of sucrose into surrounding phloem parenchyma cells (**Figure 13C**, **II**). Clearly, further studies are required to resolve whether or not phloem unloading universally includes a symplasmic passage from SE-CC complexes to phloem parenchyma cells in the release phloem zone, as found for developing seeds (Zhang et al. 2007) (**Figure 13C**, **II**).

An obvious constraint on facilitated apoplasmic unloading from SE-CC complexes is a co-requirement for a hydrolysable transported sugar (e.g., sucrose or RFO) and an invertase present within the cell walls of the release phloem zone. This combination ensures maintenance of an outwardly directed diffusion gradient for the transported sugar, due to its conversion

into a different chemical species by the cell wall invertase. Some fleshy fruits (Zhang et al. 2006; Hu et al. 2011) and pre-storage phase seeds (Zhang et al. 2007) satisfy these requirements. However, these features do not apply to plants that transport polyol through their phloem, as exemplified by apple and temperate seeds during their storage phase; these systems may well rely on energy-coupled sugar release to the seed apoplasmic space (Zhang et al. 2004, 2007) (**Figure 13C**, **II**). In this context, maximal activities of symporters retrieving sucrose from seed apoplasmic spaces into pea cotyledons or wheat endosperm cells clearly constrain phloem unloading, as shown by increases in seed dry weight of transgenic plants overexpressing these transporters (Rosche et al. 2002; Weichert et al. 2010) (**Figure 13C**, **II**).

A less obvious constraint, but an essential component of an unloading mechanism, is that unloading rates of all solutes (and particularly the major osmotic species) and water must match their rates of phloem import. Any mismatch will impact water relations of release phloem sieve tubes and, hence, translocation rates. This requirement is essentially irreconcilable if significant leakage were to occur by diffusion. To ensure matching rates of phloem import and membrane efflux, it is important to stress that membrane transport of all solutes and water needs to be facilitated. This allows activities of membrane transporters to be potentially coordinated to ensure that rates of resource import through, and unloading from, sieve tubes in the release phloem are matched (see Zhang et al. 2007).

A simple resolution to this problem is for unloading from the SE-CC complex to follow a symplasmic route and occur by bulk flow. Currently, experimental support for bulk flow, as an unloading mechanism, is limited. Such evidence is derived from experiments in which hydrostatic pressure differences between SE-CC complexes and surrounding cells were manipulated at root tips (e.g., Gould et al. 2004b; Pritchard et al. 2004). However, given that PD may represent a low hydraulic conductivity pathway, a significant pressure differential would be required to ensure the operation of an effective bulk flow delivery system.

Consistent with this prediction, large osmotic potential differences (−0.7 MPa to −1.3 MPa) between SEs of release phloem and downstream cells have been measured in symplasmic unloading pathways of root tips (Warmbrodt 1987; Pritchard 1996; Gould et al. 2004a) and developing seeds (Fisher and Cash-Clark 2000b). These differences translate into equally large differences in hydrostatic pressures, since sink apoplasmic ψ_P values approach zero (see Lalonde et al. 2003). Expulsion of sieve tube contents by bulk flow into the much larger cell volumes of phloem parenchyma cells will dissipate the hydrostatic pressure of the expelled phloem sap within these cells. This, together with frictional drag imposed by a low hydraulic conductivity PD pathway, dictates that the large differentials in hydrostatic pressure between SE-CC complexes and phloem parenchyma cells are the result rather than the cause of bulk flow (Fisher and Cash-Clark 2000b).

The above considerations draw attention to the possibility that hydraulic conductivities of PD linking SE-CC complexes with phloem parenchyma cells play a significant role in regulating phloem unloading. Interestingly, size exclusion limits of PD at these cellular boundaries appear to be unusually high in roots (60 kDa; Stadler et al. 2005), sink leaves (50 kDa; Stadler et al. 2005) and developing seeds (400 kDa; Fisher and Cash-Clarke 2000a), compared to the frequently reported value of 0.8 kDa −1.0 kDa for PD linking various cell types in ground tissues (Fisher 2000). However, size exclusion limits based on molecular weight can be misleading, and Stokes radius is a preferable measure (Fisher and Cash-Clarke 2000a). Taking this into account, PD hydraulic conductivities computed on this basis appear to be sufficient to accommodate the required rates of bulk flow out from the SE-CC complex (Fisher and Cash-Clark 2000b).

Large PD conductivities offer scope for considerable control over bulk flow across the SE-CC complex and adjoining phloem parenchyma cellular interfaces. A hint that such a system may operate is illustrated by studies on developing wheat grains. Here, imposition of a pharmacological block on sucrose uptake into the endosperm of an attached wheat grain was not accompanied by a change in sucrose concentration in cells forming the unloading pathway within maternal grain tissue (Fisher and Wang 1995). This finding points to a direct link between sucrose uptake by the endosperm and PD conductance at SE-CC-phloem parenchyma cell interfaces. How PD gating could be linked with sink demand has yet to be determined, but this would have significant implications for phloem translocation.

Phloem-imported water drives cell expansion in growing sinks (Walter et al. 2009). However, in non-expanding storage sinks, phloem-imported water is recycled by the xylem back to the parent plant body (Choat et al. 2009). This necessitates water exit across plasma membranes, irrespective of the unloading pathway, and likely depends upon movement facilitated by aquaporins (Zhou et al. 2007). Thus, except for symplasmic unloading into growing sinks, aquaporins could play a vital role in constraining rates of phloem unloading and, hence, overall phloem transport (**Figure 13C**).

Transport phloem – Far from being a passive conduit interconnecting sources and sinks

Compared to collection and release phloem, transport phloem (**Figure 13A**) extends over considerable distances of up to 100 m in tall trees. Axial flows along sieve tubes occur at astonishingly high rates, as demonstrated by estimates of specific mass transfers of approximately 500 g biomass m^{-2} sieve tube cross-sectional area s^{-1} (Canny 1975). For a time, these observations supported the notion that sieve tube cross-sectional areas

(Equation 1) were a major constraint over rates of phloem translocation (Canny 1975), a notion later dispelled by the finding that specific mass transfer rates could be elevated by an order of magnitude in modified plant systems (Passioura and Ashford 1974; Smith and Milburn 1980a; Kallarackal and Milburn 1984). More recently, a quest to obtain greater insights into sieve tube transport function (Thompson 2006) has re-ignited an interest in obtaining measures of sieve tube geometries to obtain meaningful estimates of sieve tube hydraulic conductivities (Thompson and Wolniak 2008; Mullendore et al. 2010; Froelich et al. 2011).

On the assumption that flows through sieve tube lumens and sieve pores are laminar, hydraulic conductivity (L) can be derived from the Hagen-Poiseulli Law as:

$$L = \pi r^2/8\eta l \quad (5)$$

A technically innovative and thorough quantitative plant anatomical study, undertaken across a range of eudicot herbaceous life forms, yielded estimates of sieve tube hydraulic conductivities (Mullendore et al. 2010). Values of L were found to be dominated by sieve pore radii, as predicted by Equation 5, and were inversely related with independent measures of phloem transport velocities. Such an outcome is in contradiction to the Hagen-Poiseulli Law. Together with other phloem transport anomalies, such as gradients of sieve tube hydrostatic pressures not scaling with plant size (Turgeon 2010b), these studies point to a key feature in phloem translocation likely being overlooked. As outlined below, we contend that sieve tube properties that establish conduits of exceptionally high hydraulic conductivities, combined with their ability to osmoregulate, can account for transport phloem being capable of supporting high fluxes over long (m) distances.

As indicated by Equation 5, sieve tube length (l), and most importantly, sieve pore radius (r), have a direct influence on hydraulic conductivity, along with sap viscosity (η). Sieve-tube sap viscosity varies approximately 2.5-fold across the range of measured sieve-tube sucrose concentrations (300 mM to 1000 mM sucrose) and, hence, could influence sieve tube hydraulic conductivity. The viscosity of a 600 mM sucrose solution increases approximately 2-fold from 25°C to 0°C (Misra and Varshin 1961). A controlled experiment, in which an approximate doubling of phloem sap viscosity can be achieved without impacting source or sink activities, is to gradually (to avoid shock) cool a stem zone (from 25°C to just above 0°C). Absence of any slowing of transport rates through the cooled zone (Wardlaw 1974; Minchin and Thorpe 1983; Peuke et al. 2006) argues that this range of phloem sap viscosities exerts little influence over phloem transport.

To date, studies of hydraulic conductivities deduced from sieve tube geometries have yielded ambiguous results (Mullendore et al. 2010; Froelich et al. 2011). However, indirect observations suggest that sieve tube hydraulic conductivity is unlikely to constrain phloem transport. For instance, removal of substantial proportions of transport phloem cross-sectional area from the stem had little impact on rates of translocation through the narrowed phloem zone (Wardlaw and Moncur 1976), thus indicating a considerable spare capacity for phloem transport. A spectacular example illustrating excess transport capacity is provided by a study of translocation rates through pedicels supporting developing apical fruits in racemes of *Ricinus*. Upon removal of apical fruits, and allowing exudation from their severed petiole stumps to proceed, translocation rates increased from 166 g to 3111 g biomass m^{-2} sieve-tube area s^{-1}, a response suggesting that phloem transport was sink controlled, not phloem pathway controlled (Smith and Milburn 1980a; Kallarackal and Milburn 1984).

Experimental measurements conducted using a microfluidic system simulating phloem pressure flow as well as transport properties of 'real' plants (including tall trees) also yielded results that conformed with predictions of the Münch model (Jensen et al. 2011, 2012). Studies performed on an *Arabidopsis* mutant lacking P-protein agglomerations in sieve tubes, and hence conferring higher sieve tube conductivity, established that these plants had similar transport velocities (or volume flux – see Equation 2) to WT plants (Froelich et al. 2011). Collectively, these studies support the notion that sieve-tube hydraulic conductivities do not impose a significant limitation on transport fluxes along phloem pathways, even over considerable lengths of sieve tubes. Rather, as discussed above, the majority of control may well be exercised by bulk flow through PD linking SE-CC complexes of release phloem with adjacent phloem parenchyma cells (**Figure 13C**).

Pressure-concentration waves generated by phloem unloading are transmitted over considerable distances (m) at velocities an order of magnitude higher than those of phloem translocation (Smith and Milburn 1980a; Mencuccini and Hölttä 2010). Such a signaling system is envisioned to underpin unified responses by all SE-CC complexes, comprising phloem paths from release to collection phloem, to altered resource demands by the various sinks (Thompson 2006). These responses are mediated by turgor-regulated membrane transport of sugars into SE-CC complexes; these sugars are supplied from mesophyll and axial pools for compensation within collection and transport phloem, respectively (**Figure 13A**). This mechanism results in homeostasis of hydrostatic pressure in sieve tubes along the phloem pathway (Gould et al. 2004a). The action of this pressure-concentration signaling system could account for differentials in hydrostatic pressures between collection and release phloem not scaling with transport distance, particularly in tall trees (Turgeon 2010b). In addition, such a mechanism could maintain sieve-tube sap concentrations of all solutes (Gould et al 2004a) and, hence, their rates of phloem transport.

The interconnected nature of the plant's vascular system does not appear to exert a constraint over patterns of resource flow, as demonstrated by their plasticity in response to changes in source/sink ratios (Wardlaw 1990). This leads one to the notion that the elements of the phloem function, kinetically, as a single pool of resources. A 'common' kinetic pool of phloem sap depends upon hydraulic connectivity between all functional phloem conduits. Lateral sieve areas, phloem anastomoses (Evert 2006) and intervening phloem parenchyma cells (Oross and Lucas 1985) provide conduits for resource flows to function as a common kinetic pool.

The above described transport behaviors led Don Fisher to propose a variant of the Münch pressure flow model in which he envisaged phloem systems functioning as high-pressure manifolds (Fisher 2000). High hydrostatic pressures generated by phloem loading in source leaves (Gould et al. 2005) are maintained throughout the transport phloem by osmoregulated loading in collection or re-loading by transport phloem (**Figure 13**). In the region of the release phloem, gradients in hydrostatic pressure across PD connecting SE-CC complexes to adjoining phloem parenchyma cells, in combination with PD hydraulic conductivity, control overall flows from source regions to each sink. As a corollary, relative magnitudes of PD conductivities between various sinks could control resource partitioning at a whole plant level.

The high-pressure manifold model (Fisher 2000) accounts for all known aspects of phloem transport, except direct unloading across SE-CC plasma membranes. However, as we mentioned above, conclusive evidence for this pathway forming a major phloem-unloading route has not yet been established. Indeed, symplasmic flow into surrounding phloem parenchyma cells remains a real possibility in all sinks (**Figure 13C**) and, hence, the high-pressure manifold model appears to be universally applicable.

Directing future studies to testing the phloem high-pressure manifold model

In broad terms, there is strong evidence that the high-pressure manifold model (Fisher 2000) accounts for key elements underpinning phloem transport and resource partitioning at the whole plant level. The model highlights hydraulic conductivities of PD linking release phloem SE-CC complexes with phloem parenchyma cells as the pivotal point at which phloem transport is constrained both physically and physiologically. We consider the evidence sufficiently compelling to invest significant effort in future investigations to further test the general applicability of this model. Resolving the underpinning regulatory mechanisms could open up substantial biotechnological opportunities to divert biomass flows to enhance crop yields.

Physical & Physiological Constraints on Xylem Function

The xylem of the plant vascular system transports more fluid longer distances than any other vascular tissue. The collective flow of xylem sap summed over all the plants on a watershed can exceed the total runoff in streams (Schlesinger 1997). Typically, less than 5% of the xylem water is consumed by osmotically-driven cell expansion, and less than 1% is consumed by photosynthesis. The bulk of the transported water is lost to transpiration: the water evaporates from cell wall surfaces into the intercellular air spaces of the leaves, and diffuses out into the atmosphere through open stomata. Hence, the term "transpiration stream" is used to refer to xylem sap flow. Although the transpiration stream carries nutrients, molecular signals, and other compounds from roots to leaves, and evaporative cooling can minimize overheating of larger leaves, these benefits are usually regarded as secondary to the cost of having to lose such large quantities of water in exchange for stomatal CO_2 uptake (Holtta et al. 2011). Under typical diffusion gradients, plants transpire hundreds of molecules of water for every CO_2 molecule fixed by photosynthesis. If plants could evolve a way of obtaining CO_2 without simultaneously losing water, their water consumption would be substantially reduced and water would presumably be much less of a limiting factor for their productivity.

As expected for such a poor water-for-carbon exchange rate, plants have evolved a metabolically cheap mechanism for driving the transpiration stream; otherwise, the cost of moving water could easily exceed the meager energy return. According to the well-substantiated cohesion-tension mechanism summarized in **Figure 14**, water is pulled to the site of evaporation in the leaves by the tension established within the surface of the water at the top of the water column (capillary) (Pickard 1981). The plant functions more or less as a 'water wick'. Once the 'wick' is grown, the driving force for the transpiration stream is free of charge from the plant's perspective. Most directly, the energy to drive the transpiration stream comes from the sun. However, despite its energetic efficiency, the cohesion-tension mechanism has important limitations that constrain the productivity and survival of plants. Current research questions include the evolution, physiology, and ecology of these water transport constraints.

The problem of frictional resistance to flow

The basic wicking process (**Figure 14A**) presents a physical paradox. A narrower tube is better for generating capillary at the evaporating meniscus for pulling water up, but it is worse for creating high frictional resistance to the upward flow. The maximum drop in pressure (P_{min}) created by an air-water-interface across a cylindrical pore is inversely proportional to

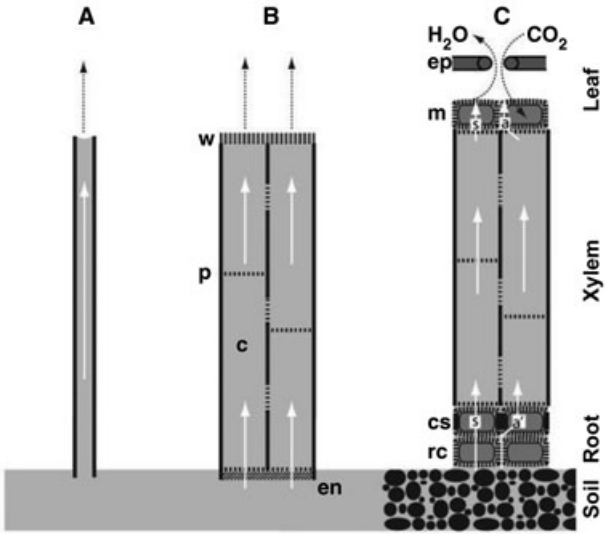

Figure 14. The cohesion-tension mechanism for transpiration-driven xylem flow.

(A) The basic hydraulic lift process: evaporation from a meniscus coupled to bulk liquid flow by capillary action.

(B) In plants, surface tension created at the surface of narrow pores within the cell walls (*w*) acts as the energy gradient to drive long-distance bulk flow through a low-resistance network of dead xylem conduits (*c*). Conduits are connected by pits (*p*), which also protect against air-entry and embolism in the inevitable event of damage (see **Figures 15**, **16**). Water is filtered through living cell membranes at the root endodermis (*en*) by reverse osmosis.

(C) Living cells (rounded rectangles enclosed by hatched cell walls) do not generate transpirational flow (*ep,* leaf epidermis; *m,* mesophyll; *cs,* Casparian strip of the endodermis; *rc,* root cortex). Water flows through them to the site of evaporation via symplastic (*s* arrows) and apoplastic routes (*a* arrows). In the root, the apoplastic route (*a'*) is interrupted by the Casparian strip. Transpiration is actively regulated by stomatal opening through which the water-for-carbon exchange occurs (from Sperry 2011).

the cylinder radius (P_{min} proportional to 1/radius), whereas the hydraulic resistivity (resistance per unit length) through the cylinder increases with 1/radius^4. The higher the resistivity, the lower the flow rate at which P drops to P_{min}, pulling the meniscus down the tube and drying out the wick. The evaporating menisci of the plant are held in the nanometer-scale pores of primary walls facing internal (intercellular) air spaces. Although ideal for generating a potentially substantial driving force for pulling up the transpiration stream, the high resistivity of these pores to bulk flow limits the distance and rate of water flow. Hence, high flow resistivity of parenchymatous plant ground tissue was a major factor limiting the size of non-vascular (pre-tracheophyte) plants.

With the evolution of xylem conduits, the wick paradox was solved: maximum capillary action at nano-scale cell wall pores is coupled to minimum bulk flow resistivity in micro-scale xylem conduit lumens for carrying the transpiration stream over most of the soil-to-leaf distance (**Figure 14B**). The conduits are dead cell wall structures that resist collapse by having lignified secondary walls. They form a continuous apoplasmic pipeline from terminal leaf vein to root tip, propagating the tensional component to the supply of water in the soil. As water is pulled into the stele of the absorbing root tissues, it is forced across the endodermal membrane by reverse osmosis, providing a mechanism for filtration and selective uptake (**Figure 14B**, en). Like cell wall water, the soil water is held by capillary and absorptive forces. In short, the cohesion-tension mechanism is a "tug-of-war" on a rope of liquid water between capillary forces in soil vs. plant apoplasm. Living protoplasts do not participate in driving the transpiration stream, but draw from it by osmosis during cell expansion growth and to stay hydrated and turgid (**Figure 14C**).

Low resistivity xylem facilitates larger plants and higher flow rates (equals greater photosynthetic productivity), and xylem evolution is a history of innovations for presumably moving water more efficiently. The increasing literature on the topic is beyond the more physiological emphasis of this section of the current review, but one example will suffice. The rise of high-productivity angiosperms appears to coincide with the evolution of greater vein density within leaves, which minimizes the distance the transpiration stream flows in high-resistance parenchymatous ground tissue (Boyce et al. 2009). Presumably, the evolutionary pressure for maximizing hydraulic efficiency varied over geological time scales, being less during periods of higher atmospheric CO_2 and arid (dry) conditions, and increasing during periods of low CO_2 and mesic (wet) conditions (Boyce and Zwieniecki 2012).

The reason that lower frictional resistance correlates with greater photosynthetic rate is because it also correlates with greater diffusive conductance of the stomatal pores (Meinzer et al. 1995; Hubbard et al. 2001). The physics of the xylem conduit does not account for this coupling between low flow resistance and high diffusive conductance (equates to high evaporation rate). As long as the integrity of the xylem conduit remains constant, its evaporation rate is essentially independent of its internal liquid phase flow resistance. The observed coupling must result from a physiological response of the plant.

The simplest explanation for the coordination of low hydraulic resistance and high diffusive conductances is that stomata are responding in a feedback manner to some measure of leaf or plant water status (Sperry 2000; Brodribb 2009). Increased plant hydraulic resistances result in a greater tensional component (i.e., more negative values of ψ_P) at a given transpiration rate and soil water status. If the ψ_P falls below some regulatory set-point (which need not be constant), hydraulic or chemical

signals are sent to the stomatal complex, reducing the stomatal aperture and transpiration rate, which causes the ψ_P to rise back above the set-point. This ψ_P feedback is at least broadly consistent with most observed stomatal behavior in response to hydraulic resistance, as well as to evaporative demand and soil moisture stress (Oren et al. 1999; Brodribb 2009; Pieruschka et al. 2010).

Recent evidence indicates that the ancestral feedback was entirely passive, as indeed it appears to still be in many seedless vascular plants (Brodribb and McAdam 2011). Accordingly, as ψ_P becomes more negative, guard cell turgor drops without any chemical signaling or active osmotic adjustment. Ensuing stomatal closure reduces the transpiration rate, which causes ψ_P to stop falling and rise back up. Only with the divergence of the seed plants 360 Mya did apparently active signaling evolve, which involves ψ_P sensing mechanisms, triggering of abscisic acid and other chemical signaling molecules, and active osmotic adjustment via ion pumps at the stomatal complex (McAdam and Brodribb 2012). The details of how this active feedback is achieved, the extent of active vs. passive mechanisms, the actual sites of evaporation within the leaf, the sites of water potential sensing, and the extent of hydraulic coupling between the stomatal complex and xylem are basic questions that are still poorly understood, and are the subject of considerable research and debate (Pieruschka et al. 2010; Mott and Peak 2011).

Regardless of the feedback details, plants appear to have evolved to regulate ψ_P at the expense of sacrificing photosynthesis via reduced stomatal diffusive conductance. Presumably, this response avoids deleterious consequences of excessively negative ψ_P. Certainly most physiological processes are more energy-demanding at more negative ψ_P: the xylem conduit has to be stronger, and protoplasmic osmotic concentrations have to be greater. The implication is that there is some optimal midday ψ_P, in so far as it maximizes the cost/benefit margin of water transport vs. CO_2 processing (Holtta et al. 2011). *A priori*, the optimal ψ_P would differ across habitats. For example, it would need to be more negative in drier habitats where plants have to pull harder to extract soil water as compared to wetter habitats. A full understanding of the costs associated with xylem transport requires detailed consideration of how xylem structure relates to its role in the water conducting process.

Confining embolism with inter-conduit pitting

While the evolution of xylem conduits solved the so called "wick" paradox, a new problem was created: the low-resistivity xylem conduits are necessarily too wide to generate, in and of themselves, much of a tension, i.e., a negative ψ_P value. In the inevitable event that a conduit becomes damaged and exposed to air, the surface tension present in the new meniscus spanning the conduit lumen is too weak to resist retreat (P_{min} is not negative enough), and so water is pulled from this specific conduit into neighboring conduits and becomes embolized; i.e., it eventually is filled with N_2, O_2, CO_2 and water vapor gases. Thus, capillary rise within the xylem conduit lumen cannot do the job of pulling up the transpiration stream. For the system to function, the xylem conduits must be primed by being water filled from inception, as they are, having developed from living cells. Nevertheless, there must be a means of limiting the spread of embolism when inevitably a conduit is damaged, even if by normal developmental events such as abscission of parts or protoxylem rupture.

The problem of embolism is mitigated most fundamentally by dividing the fluid conducting space into thousands of overlapping and inter-connected conduits (**Figure 14B, C**). Each one embolizes as a unit because the inter-connections consist of porous partitions (inter-conduit pits) fine enough to trap and hold an air-water meniscus against a sufficiently negative ψ_P to minimize further gas propagation (**Figure 14B**, p). This multi-conduit system necessarily compromises hydraulic conductance because of the added resistance to flow through the inter-conduit pitting. Presumably, the lowest hydraulic resistance would be achieved by a single branching tube akin to the animal positive-pressure cardiovascular system. But a tensional system of such design would fail completely from a single point of air entry without any partitions to check the influx of gas.

The presence of inter-conduit pitting is of great consequence for xylem functioning. The distribution of pitting and the structure and chemistry of individual pits influence both the flow resistance through the xylem and the P_{min} for the xylem system (Choat et al. 2008). Although there is tremendous variation in inter-conduit pit structure across lineages, their basic structure has three elements held in common (**Figure 15**). As water flows from one conduit lumen to another, it passes through a pit aperture in the secondary wall, which opens into a usually wider pit chamber. Spanning the chamber is a porous pit membrane through which the water filters before passing out the downstream aperture. The pit membrane is the modified primary cell walls plus middle lamella of the adjacent conduits. There is no cell membrane or protoplasm in the dead but functioning conduit.

Hydrolysis during xylem cell death is thought to remove all hemicelluloses and a debatable portion of pectins from the pit membrane, leaving a porous cellulosic mesh of microfibrils (Butterfield 1995). However, atomic force microscopy suggests that the microfibrillar structure lies beneath a non-porous coating of amorphous non-fibrillar material (Pesacreta et al. 2005). Pit membrane chemistry, structure, and development are crucial to understanding the frictional resistance to flow as well as the ability of the xylem to sustain significant tensions in the water column (Choat et al. 2008; Lee et al. 2012).

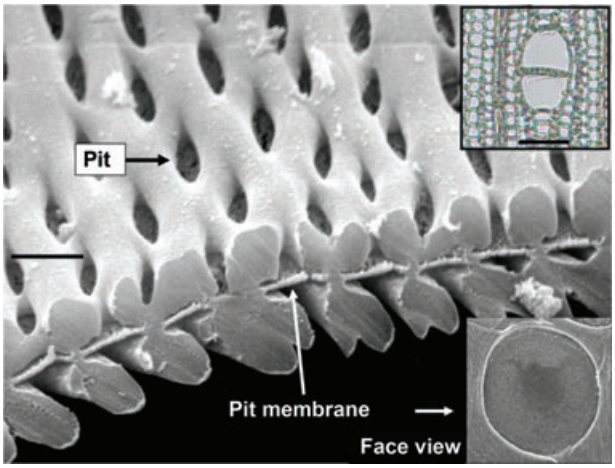

Figure 15. Inter-vessel pit structure in angiosperm wood.

Upper-right insert shows brightfield image of two overlapping vessels. Their common wall is studded with inter-vessel pits as shown in the main scanning electron microscopy (SEM) image where an inter-vessel wall has been sectioned to show individual pit structure. Pits consist of openings in the secondary wall (apertures) leading to pit chambers that are spanned by a pit membrane which is the modified primary cell wall and middle lamella of the adjacent vessel elements. The lower-right insert shows an SEM face view of the micro-porous pit membrane. Scale bars: 5 μm and 30 μm for the upper inset. Micrographs courtesy of Fredrick Lens, Jarmila Pittermann, and Brendan Choat.

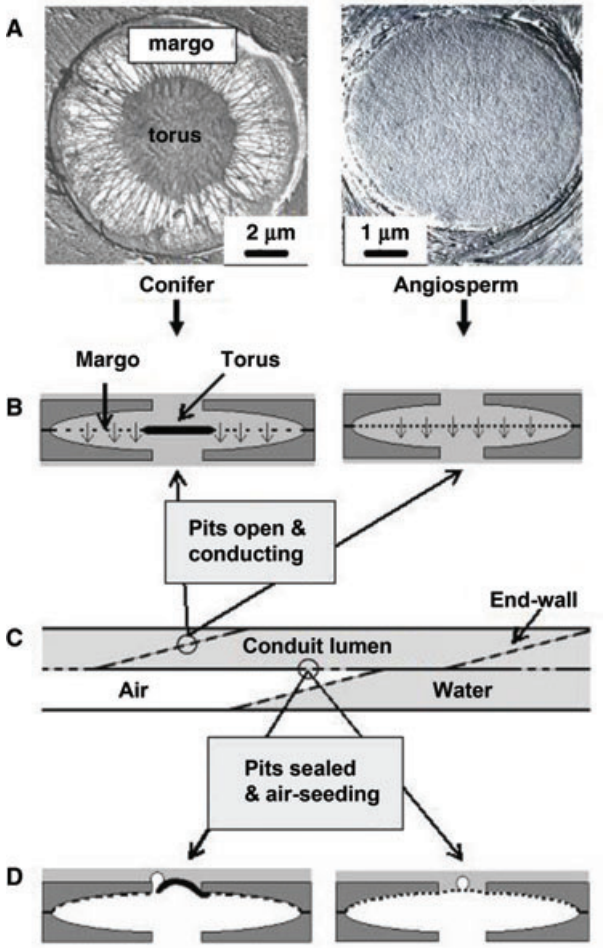

Figure 16. Structure and function of inter-conduit pits in conifer tracheids (left) and angiosperm vessels (right).

(A) Pit membranes, face view.
(B) Side view schematic of membranes within the pit chamber formed by the secondary walls. Pits open and functioning in water transport.
(C) Schematic of pit location within conduit network.
(D) Side view of pits in sealed position showing proposed air-seeding process (from Pittermann et al. 2005).

Inter-conduit pits add substantial flow resistance to the xylem conduit lumen. An unobstructed lumen conducts water as efficiently as an ideal cylindrical capillary tube of the same diameter (Zwieniecki et al. 2001a; Christman and Sperry 2010). The most extensive survey indicates that adding inter-conduit pits increases flow resistivity over that of an unobstructed lumen by an average factor of 2.8 in conifers with unicellular tracheids and 2.3 in angiosperms with multicellular vessels (Hacke et al. 2006; Pittermann et al. 2006a). The lower number for vessels is not surprising given that they are roughly 10 times longer than a tracheid of the same diameter (Pittermann et al. 2005), thereby spacing high resistance pits further apart and reducing the length-normalized resistance (resistivity). What is surprising is that the greater length of vessels does not have more of an effect: if inter-vessel pits have the same area-specific pit resistance as inter-tracheid pits, placing the end-walls 10 times further apart should increase lumen resistivity by less than a factor of 1.18. The higher observed factor of 2.3 indicates that inter-vessel pits have higher flow resistance than inter-tracheid ones, a difference consistent with anatomy and estimations based on modeling (Pittermann et al. 2005).

Inter-vessel pits have nano-porous "homogeneous" pit membranes (pores usually $<$ 100 nm) (Choat et al. 2008), or often with no pores detectable, whereas inter-tracheid pits of conifers have a highly porous margo (pores $\gg$ 500 nm) (Petty and Preston 1969) peripheral to a central thickened torus (**Figure 16A**, left). The greater porosity of the margo decreases the area-specific pit resistance by an estimated 59-fold relative to inter-vessel pits of angiosperms (Pittermann et al. 2005). The homogenous-type pit membrane is presumably ancestral, and the implication is that the evolution of efficient torus-margo pitting, within in the gymnosperm lineage, was as hydraulically advantageous as the evolution of vessels in angiosperms.

Pit membrane chemistry interacts with xylem sap chemistry to influence xylem flow resistance in a very complex and poorly understood manner. According to the "ionic effect", increasing the concentration of KCl up to 50 mM (encompassing the physiological range) can decrease resistivity anywhere from 2% to 37% relative to pure water, depending on the angiosperm species and even the season (Nardini et al. 2011b). The KCl effect is much less or even negative in the already low-resistance torus-margo pits of conifer species (Cochard et al. 2010b). Furthermore, this KCl effect can be reduced or eliminated in the presence of as little as 1 mM Ca^{2+} in some species and conditions (Van Iepeeren and Van Gelder 2006), but not in others (Nardini et al. 2011b). Skepticism about the importance of the phenomenon *in planta* (Van Iepeeren 2007) has been answered with observations indicating KCl-mediated decreases in resistivity associated with embolism and exposure of branches to sunlight (Nardini et al. 2011b). Interestingly, adaptive adjustments in KCl concentration may be mediated by xylem-phloem exchange (Zwieniecki et al. 2004).

The ionic effect has been localized to the pit membranes, but the mechanism remains unknown. A "hydrogel" model implicates ionic shrinkage of pit membrane pectins or equivalent hydrogel polymers, and, hence, a widening of membrane pores (Zwieniecki et al. 2001b). However, recent observations with atomic force microscopy do not support a pore-widening effect. Although KCl was observed to thin the membrane, pores were not observed, suggesting that the decrease in resistance resulted from membrane thinning and perhaps increased permeability of non-porous gel material (Lee et al. 2012). The extent of pectins or similar gel materials in pit membranes appears to be highly variable across species, perhaps underlying the extreme variation in the ionic effect (Nardini et al. 2011b). Uncertainty about the extent of hydrogel components of pit membranes has led to an alternative (and perhaps complementary) hypothesis that ions increase permeability by reducing the diffuse-double layer of cations lining negatively charged nano-scale pores in the membrane (Van Doorn et al. 2011b). All of these hypotheses are consistent with a minor effect in torus-margo pit membranes, with their large micro-scale pores between cellulosic strands having presumably minimal pectin content.

Although inter-conduit pits have the disadvantage of adding substantial flow resistance, they perform the highly advantageous function of trapping an air-water meniscus and minimizing the embolism event such that it does not compromise the conducting system (**Figure 16B, C**). The homogenous pits of angiosperm vessels have pores narrow enough to trap the meniscus with a P_{min} negative enough to hold against a substantial range of negative ψ_P values (**Figure 16D**). The torus-margo pits function somewhat differently. The wider margo pores cannot sustain a very negative P_{min}, but they can generate just enough pressure difference to aspirate the solid torus against the pit aperture on the water-filled side (Petty 1972). In this way, the torus can seal off the pit with a sufficiently negative P_{min} to minimize air passage (**Figure 16D**).

While inter-conduit pits minimize the propagation of embolism, as the next section indicates, they nevertheless play a major role in limiting the tensional gradient that can be generated by the cohesion-tension mechanism.

Limits to negative ψ_P values: the problem of cavitation

Periodically, the cohesion-tension mechanism comes under question for its prediction of liquid pressures that fall below the vapor pressure of water, and also below pure vacuum for a gas (Canny 1998; Zimmermann et al. 2004). A tree 30 m tall requires a ψ_P of -0.3 MPa on its stationary water column just to balance the gravity component. To this we need to add, say, -0.3 MPa to balance a favorable soil water potential of -0.3 MPa. Finally, we need to add the typical $\Delta\psi_P$ of -1 MPa needed to overcome frictional resistance under midday transpiration rates. The required ψ_P totals -1.6 MPa. At sea level and 20°C, a vapor pressure of only -0.098 MPa will bring water to its boiling point, and -0.1013 MPa corresponds to pure vacuum for a gas. Clearly, for the cohesion-tension mechanism to operate, transition from the liquid phase to the vapor phase must be suppressed, and the xylem sap must remain in a metastable liquid state. The xylem sap is in effect super-heated, although "super-tensioned" is more descriptive. The liquid water column becomes analogous to a solid whose strong atomic and intermolecular bonds allow it to be placed under tension; i.e., water is a tensile liquid!

The concept of metastable water is foreign to the macroscopic world of normal human experience, hence the cohesion-tension skeptics. Water boils at 100°C, and vacuum pumps become gas-locked at or above -0.098 MPa. But in these familiar cases, the phase change to vapor (cavitation) is nucleated by contact with foreign agents that destabilize the inter-molecular hydrogen-bonding of liquid water (Pickard 1981). Such "heterogeneous nucleation" of cavitation is typically triggered by minute and ubiquitous gas bubbles in the system. When care is taken to minimize such heterogeneous nucleation, liquid water can develop substantially metastable negative ψ_P values. Theoretical calculations, based on equations of state for water, put the limiting ψ_P at homogeneous cavitation (where energy of the water molecules themselves is sufficient to trigger the phase change) below -200 MPa at 20°C (Mercury and Tardy 2001). Experiments with a variety of systems ranging from centrifuged capillary tubes to water-filled quartz crystals have reached values well below -25 MPa, with some as low as -180 MPa and approaching the theoretical limit (Briggs 1950; Zheng et al. 1991). Such values dwarf even the most negative ψ_P in plants, which is about -13 MPa (Jacobson et al. 2007); more typical plant ψ_P values are less negative than -3 MPa.

There is abundant evidence that cavitation "pressures" in plants are negative enough for the cohesion-tension mechanism to operate. Such pressures are determined from a "vulnerability curve" which usually plots the hydraulic conductivity (reciprocal of resistivity) of the xylem (often as a percentage loss from maximum) as a function of the ψ_P value in the xylem sap. Curves are generated in several ways, but the centrifuge method is commonly used because of its rapidity (Alder et al. 1997; Cochard et al. 2005). Stems or roots are spun in a custom centrifuge rotor that places their xylem under a known tension at the center of rotation. The conductivity is measured either during or between spinning, and the experiment is continued until the conductivity has dropped to negligible values, thus indicating complete blockage of flow by cavitation. Typical species-specific variation is shown in **Figure 17A**, and **Figure 17B** compares the ψ_P value causing complete loss of xylem conductivity with the minimum ψ_P values measured in nature for 102 species (Sperry 2000). Clearly, the xylem of some species cavitates much more readily than others, but these vulnerable species also do not develop very negative ψ_P values in nature. Across the board, the minimum ψ_P is generally less negative than the value of ψ_P at zero conductivity, as is required by the cohesion-tension mechanism.

Although the centrifuge method is widely accepted for conifers and for short-vesseled angiosperms, there is some controversy about its ability to measure the vulnerability of long-vesseled taxa where many conduits can exceed the length of the spinning conductivity segment. Response curves in these taxa indicate that a significant number of these large vessels cavitate at very modest negative ψ_P values. This pattern is seen in two of the curves shown in **Figure 17A** (open symbols). Comparisons with other methods have verified this type of curve in many (Christman et al. 2012; Jacobson and Pratt 2012; Sperry et al. 2012), but not in all cases (Choat et al. 2010; Cochard et al. 2010a), and resolving the matter requires further research.

A major cause of the cavitation in plant xylem is air-seeding through the inter-conduit pits that normally are responsible for confining gas embolism (Crombie et al. 1985; Sperry and Tyree 1988; Sperry and Tyree 1990; Jarbeau et al. 1995). The P_{min} of inter-conduit connections is easily measured from the positively applied gas pressure that just breaches their seal (Christman et al. 2012). From a variety of techniques employed across a wide range of species, the P_{min} range of inter-conduit pitting is generally indistinguishable from the range of ψ_P values that cause cavitation. In consequence, vulnerability curves can usually be reproduced using positive gas pressures rather than placing the xylem sap under tension (Cochard et al. 1992). The prevailing model of cavitation nucleation is that when the ψ_P in the transpiration stream drops to the P_{min} of the inter-conduit seal against adjoining embolized vessels, a gas bubble is pulled into the transpiration stream which then nucleates cavitation. The sap ψ_P in the cavitated conduit immediately rises to 0, and the sap is very quickly drained out of the conduit by the surrounding transpiration stream until the entire conduit becomes filled with water vapor and air, the exact composition of the embolism depending on diffusive exchange with the surrounding tissue (Tyree and Sperry 1989). According to this model, cavitation and embolism can propagate from conduit-to-conduit via pit failure.

Considerable attention has been given to how the structure of inter-conduit pitting relates to its function in cavitation resistance. In conifer tracheids, where torus aspiration seals the pits, it has been proposed that air-seeding occurs when the pit membrane is stretched sufficiently to displace the torus from the aperture, exposing part of the margo through which air can readily pass (**Figure 16D**). Displacement has been observed microscopically, and conifers that are more resistant to cavitation generally have less flexible pit membranes, and can have a torus that covers the aperture with greater overlap (Sperry and Tyree 1990; Domec et al. 2006; Hacke and Jansen 2009). An alternative model is that air seeding occurs by displacement of the air-water-interface between the torus and the pit chamber wall. In some conifers, air-seeding could also occur through pores in the torus. In support of these mechanisms is a dependence of P_{min} on the sap surface tension (Cochard et al. 2009; Holtta et al. 2012; Jansen et al. 2012). However, it is not clear whether sap solutions that lower the surface tension also alter pit membrane flexibility and, hence, the ease of torus displacement. It is also unclear whether the torus-margo membrane can de-aspirate to function a second time if the embolized tracheids refill.

In angiosperm inter-vessel pits, air seeding probably occurs by displacement of the air-water meniscus from pores in their homogenously nano-porous pit membranes (**Figure 16D**), but beyond this, the details are surprisingly complex and ambiguous. Air seeding at pores is implied by the predicted sensitivity to surfactants and correspondence between pore dimensions, sized by particle penetration, and air-seeding pressures (Crombie et al. 1985; Sperry and Tyree 1988; Jarbeau et al. 1995). The pores may be pre-existing in the membrane, or perhaps created or enlarged by the partial or complete aspiration of the membrane that likely precedes the air-seeding (Thomas 1972). A major role of membrane strength is indicated by the effects of removing Ca^{2+} from the membrane using various chelators such as oxalic acid. These treatments do not alter surface tension or hydraulic conductivity, but they can dramatically increase membrane flexibility and vulnerability to cavitation (Sperry and Tyree 1988; Sperry and Tyree 1990; Herbette and Cochard 2010). Further support for the importance of membrane mechanics is the cavitation "fatigue" phenomenon wherein xylem becomes more vulnerable to cavitation after having been cavitated and

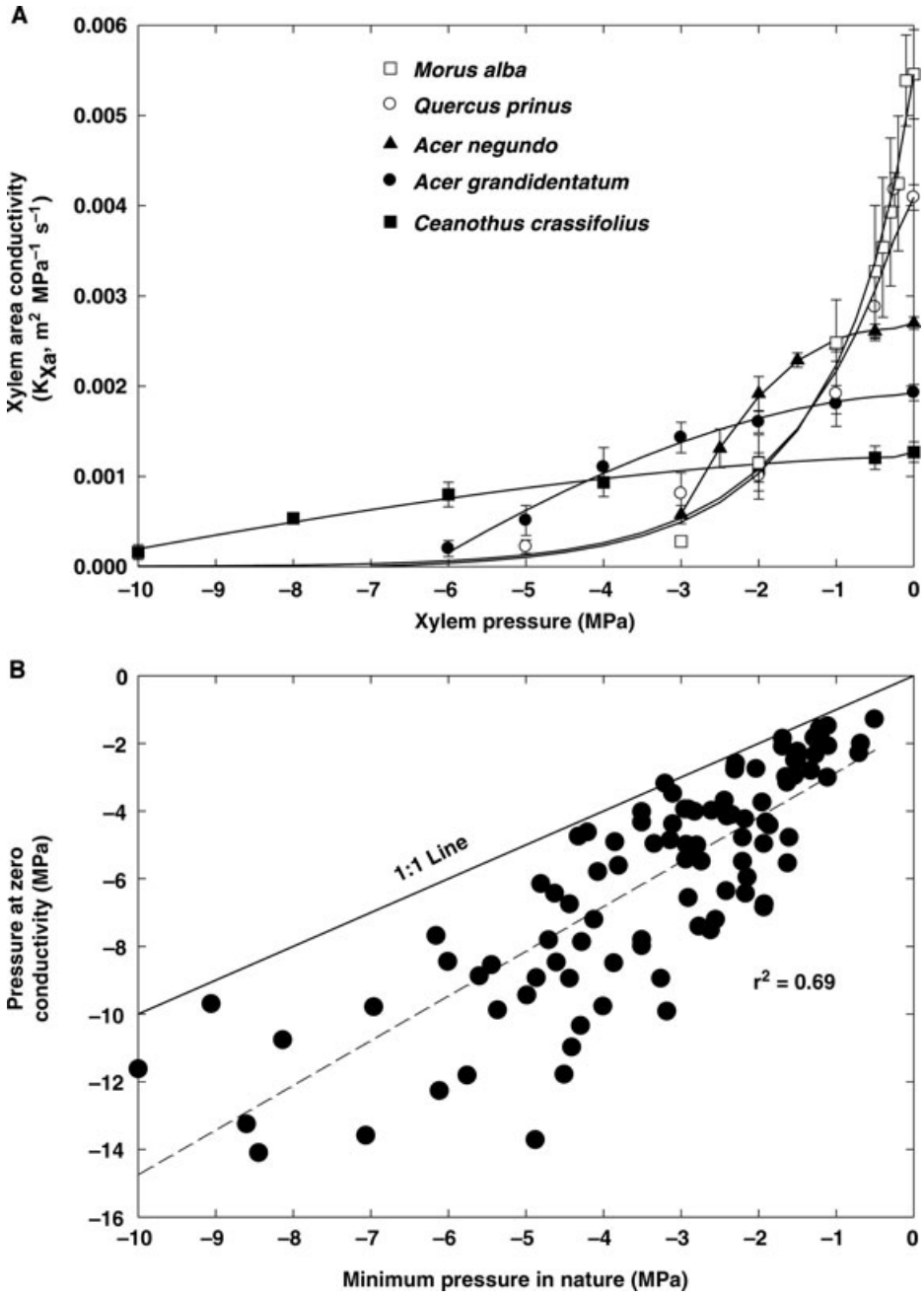

Figure 17. Vulnerability of xylem to cavitation by negative pressure.

(A) Vulnerability curves for five species showing the drop in xylem hydraulic conductivity (normalized by stem cross-sectional area) as the xylem pressure becomes more negative. Curves were generated using the centrifugal force method. Species can differ considerably in their maximum hydraulic conductivity (x axis intercept) and how readily they lose it to cavitation (from Hacke et al. 2006).

(B) Xylem pressure at zero hydraulic conductivity from cavitation (the y intercept of a, above) vs. the minimum pressure observed in nature for 102 species (from Sperry 2000).

re-filled. The implication is that mechanical stress has plastically deformed the membrane to make it more porous. Cavitation fatigue is potentially reversible *in vivo* and with artificial xylem saps, suggesting the stressed and air-seeded pit membrane can be restored back to its normal form (Hacke et al. 2001b; Stiller and Sperry 2002).

The possibility that pores are created by mechanical stress on the pit membrane is also consistent with the typical rarity of

observable pores of predicted air-seeding size in non-stressed membranes (Choat et al. 2008), as well as the tendency for pit membranes to be thicker, pit chambers shallower (less deflection), and apertures proportionally smaller in cavitation-resistant species (Choat et al. 2008; Jansen et al. 2009; Lens et al. 2010). Furthermore, the KCl-induced decrease in membrane flow resistance does not translate into increased vulnerability to cavitation (Cochard et al. 2010b). This could mean there are few to no pores in the non-stressed water conducting pit membrane, with water penetrating the hydrated gel phase. Alternatively, this could support the diffuse-double layer hypothesis for the KCl effect which does not require changes in pore dimensions (Van Doorn et al. 2011b). The apparent interaction between membrane stress and air-seeding would be largely eliminated for "vestured" pits where, in some plant lineages, the membrane is supported by outgrowths from the pit chamber wall, a factor that must lie behind the elusive adaptive significance of these structures (Jansen et al. 2001; Choat et al. 2004).

An additional complexity in linking cavitation resistance to pit structure is a seemingly inescapable role for probability. A single inter-conduit seal consists of hundreds if not thousands of individual pits. Not all of these pits will be created equal, and it only takes one leaky pit to air-seed the cavitation. According to the "rare pit" hypothesis, the more pits that constitute the seal, the weaker the seal will be, because by chance the leakier will be the leakiest pit (Wheeler et al. 2005). This will be true whether the air-seeding pores are pre-existing, or are created by mechanical stress. This hypothesis is supported by the general trend for larger vessels to be more vulnerable to cavitation (because they would have more pits), and by a correlation between the extent of inter-conduit pit area and increasing vulnerability (Hacke et al. 2006; Christman et al. 2009; Christman et al. 2012). It seems safe to conclude that both pit quantity and pit quality interact to set the ψ_P value at which air-seeding occurs and, hence, the cavitation resistance (Lens et al. 2010).

Because cavitation appears to spread from conduit to conduit, the three dimensional connectivity of the conduit network should have an additional impact on cavitation resistance. Woody species differ considerably in the extent of overlap or connectivity between individual conduits (Carlquist 1984). Modeling studies suggest that greater connectivity would tend to facilitate the spread of embolism, whereas more limited connectivity would tend to confine it (Loepfe et al. 2007). However, limited comparative data do not always support this prediction. A greater vessel grouping index (higher connectivity) in some lineages correlates with higher cavitation resistance rather than enhanced vulnerability (Lens et al. 2010). This trend supports the earlier notion that the greater redundancy provided by high connectivity would be advantageous for minimizing the effect of embolism in xeric habitats (Carlquist 1984).

The evident complexity that links xylem conduit structure to cavitation resistance comes from the multitude of variables involved: features at the individual pit level (e.g. membrane porosity, thickness, Ca^{2+} content, mechanical properties) can be balanced by features at the inter-conduit wall scale (e.g., pit number), which in turn can be balanced by features at the conduit network level (e.g., conduit connectivity). Thus, the same cavitation resistance is likely to be achieved by multiple structural combinations.

Freeze-thaw associated cavitation

Cavitation can also be induced by freeze-thaw cycles and likely by a "thaw expansion" nucleating mechanism (Pittermann and Sperry 2006) (**Figure 18A**). Freezing of the xylem sap in nature usually occurs under conditions where transpiration is minimal. Thus, xylem blockage by ice formation would normally not result in ψ_P becoming more negative. Instead, ψ_P would likely become less negative, or even become positive because of the expansion of ice. However, dissolved gases in the sap are insoluble in ice, and under typical freezing conditions will form bubbles in the middle of the conduit. On thawing, if these bubbles do not dissolve fast enough they can nucleate cavitation if the thawed sap ψ_P is sufficiently negative. Hence, cavitation would occur during the thawing rather than the freezing phase, a prediction supported by experimental observation (Mayr and Sperry 2010).

Just as for cavitation by water stress, there is considerable variation between species in their vulnerability to freeze-thaw cavitation. It is scarcely detectible in some species regardless of the negative sap ψ_P values, and others are blocked completely by a single freeze-thaw event at ψ_P values close to zero (Davis et al. 1999) (**Figure 18B**). However, unlike the water stress situation, there appears to be a single structural variable of over-riding importance for determining vulnerability to freeze-thaw cavitation. That variable is the xylem conduit lumen diameter: wider conduits are uniformly more susceptible to cavitation than narrower ones, regardless of whether the conduit is a conifer tracheid or angiosperm vessel (**Figure 18B**). The simplest explanation is that wider vessels form larger bubbles during freezing because of their greater water volume, and large bubbles take longer to re-dissolve and, hence, are more likely to nucleate cavitation, post-thaw. As the thaw-expansion model predicts, a more negative ψ_P post-thaw, or a more rapid thawing rate, will also induce more cavitation at a given conduit diameter (Langan et al. 1997; Pittermann and Sperry 2003, 2006). Similarly, the amount of embolism should decrease with a greater rate of freezing, which reduces gas bubble size (Sevanto et al. 2012).

In some species, the amount of embolism increases with the minimum freezing temperature, an observation not necessarily predicted from the thaw-expansion mechanism (Pockman and

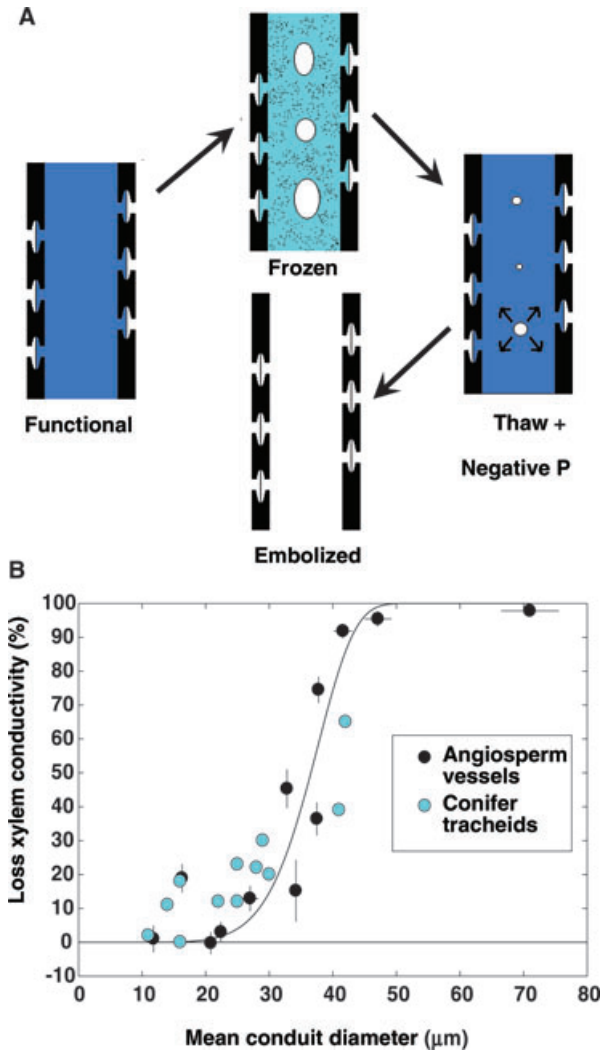

Figure 18. Vulnerability of xylem to cavitation by freeze-thaw events.

(A) The "thaw expansion" mechanism for cavitation by freezing and thawing. Freezing of a sap-filled functional vessel creates gas bubbles in the ice-filled frozen conduit. If bubbles persist long enough after thawing, and negative pressures are low enough, they will trigger cavitation and result in an embolized conduit (from Sperry 1993).

(B) Loss of hydraulic conductivity caused by a single freeze-thaw cycle at -0.5 MPa versus the average conduit lumen diameter. Data are species averages for angiosperm vessels (black) and conifer tracheids (blue) (from Pittermann and Sperry 2003).

Sperry 1997; Ball et al. 2006; Pittermann and Sperry 2006). It is possible that the lower ice temperature and consequent tissue dehydration creates, locally, a more negative sap ψ_P, post-thaw (Ball et al. 2006), or perhaps causes tissue damage that nucleates cavitation, post-thaw. Acoustic emissions are often detected during the freezing phase, which could indicate that at least some cavitation occurs prior to the thawing of tissue (Mayr and Zublasing 2010). However, experiments indicate no loss of conductivity when stems frozen under negative ψ_P conditions are thawed at atmospheric pressure; the conductivity only drops when the thaw occurs under negative ψ_P values (Mayr and Sperry 2010). It is possible that other phenomena besides conduit cavitation are causing freezing-associated acoustic emissions. Importantly, not all embolism events during winter are necessarily caused by freeze-thaw cycles. Sublimation and cavitation by water stress in thawed and transpiring crowns with frozen boles or soil represent other potential causes (Peguero-Pina et al. 2011).

Negative xylem sap ψ_P and conduit collapse

The cohesion-tension mechanism requires conduit walls that are sufficiently rigid to withstand collapse by the required negative ψ_P values. Hence, the evolution of secondary walls and lignification necessarily paralleled the evolution of xylem tissues. While many factors contribute to the strength of conduit walls to prevent implosion, a dominant variable is the ratio of wall thickness to conduit lumen radius. This ratio tends to increase with cavitation resistance, as expected from concomitantly more negative sap ψ_P values. A higher thickness-to-span ratio also increases wood density, consistent with the tendency for greater wood density in more cavitation-resistant trees that generally experience more negative ψ_P values (Hacke et al. 2001a; Domec et al. 2009).

Estimates of wall strength give an average safety factor from implosion of 1.9 in woody angiosperm vessels and 6.8 in conifer stem tracheids (Hacke et al. 2001a). The lower value for vessels presumably reflects their minimal role in mechanical support of the tree, a function performed by wood fibers. However, conifer tracheids must be additionally reinforced because they not only have to hold up against negative sap ψ_P, but they also support the tree itself. Interestingly, not all conduits avoid implosion, as it has been observed in the axial tracheids of pine needles (Cochard et al. 2004), transfusion tracheids of podocarps (Brodribb and Holbrook 2005), and metaxylem vessels in maize (Kaufman et al. 2009). In each case, the collapse was apparently reversible. Not unexpectedly, implosion is also observed in conduits of lignin-deficient mutants (Piquemal et al. 1998).

Trade-offs between efficiency and safety

The cohesion-tension mechanism, and its limitation by cavitation and conduit collapse, suggest potential trade-offs in the xylem conduit structure for minimizing flow resistance on the one hand (efficiency), and sustaining greater negative ψ_P without cavitation or conduit collapse on the other hand (safety). With respect to greater resistance to collapse, large increases

in the thickness-to-span ratio would be more readily achieved by narrowing the lumen, because walls arguably have a limited maximum thickness (Pittermann et al. 2006b). Tradeoffs arise because narrower lumens would have exponentially greater flow resistance, and higher thickness-to-span increases construction costs. Greater resistance to cavitation by freeze-thaw cycles also comes at the expense of narrower lumens, and consequently, higher flow resistance per conduit (Davis et al. 1999). Resistance to freeze-thaw embolism can, in turn, trade-off with photosynthetic capacity in evergreen species (Choat et al. 2011).

Enhanced resistance to cavitation by water stress also tends to be associated with higher flow resistances, as seen in the collection of vulnerability curves shown in **Figure 17A**. The more vulnerable species tend to have higher initial hydraulic conductivities than the more cavitation-resistant ones. The basis for this association is not straightforward because so many variables interact to determine a species' vulnerability curve. While intuitively one might expect that pits which are more resistant to air-seeding would also have a greater flow resistance (Holtta et al. 2011), the data suggest otherwise: membrane flow resistance is uncoupled from membrane air-seeding resistance. Estimates of pit flow resistances across several angiosperm and conifer species showed no relationship with cavitation resistance (Pittermann et al. 2005; Hacke et al. 2006). Furthermore, the drop in flow resistance via the ionic effect on pit membranes had no effect on cavitation resistance (Cochard et al. 2010b). Instead, the efficiency-safety trade-off may arise at the whole-conduit and conduit-network scale. If the rare pit hypothesis were correct, greater cavitation resistance would require fewer pits per conduit, which would generally correspond to narrower and shorter conduits of consequently greater flow resistance (Wheeler et al. 2005). And if reducing the connectivity of a conduit (the number of conduits it contacts) limits the spread of embolism, as expected, the lower connectivity may translate to lower conductivity at the network scale (Loepfe et al. 2007).

The flow resistance penalty of narrower and shorter conduits can be compensated by increasing their number per wood area (Hacke et al. 2006). This "packing" strategy is exemplified by conifers which devote over 95% of their wood volume to tracheids and, thus, achieve similar whole stem hydraulic conductivity as angiosperms whose generally wider and longer vessels occupy only a small fraction of wood space, most of which is devoted to structural fibers and storage parenchyma. The packing strategy exemplifies how trade-offs at one level of structure can be compensated for at another scale, vastly complicating the adaptive interpretation of wood structure and function.

Trade-offs of one sort or another presumably underlie the observed correlation between cavitation resistance and the range of native sap ψ_P values (**Figure 17B**). Accordingly, mesic-adapted species that experience less negative xylem ψ_P are vulnerable to cavitation because being overly resistant would cost them in terms of greater flow resistance and vascular construction costs. Conversely, xeric-adapted species that periodically endure more negative ψ_P values must be more resistant to cavitation, and their consequently greater flow resistance and construction costs competitively exclude them from more mesic habitats. The result of this adaptive scenario is that species tend to be only as resistant to cavitation as they have to be for the native ψ_P range they experience over their lifespan (Maherali et al. 2003; Markesteijn et al. 2011).

The limiting process of cavitation naturally constrains the xylem ψ_P over which productivity can be sustained. Indeed, an important adaptive advantage of stomatal regulation of ψ_P is to keep it from reaching such negative values that would induce excessive cavitation (Tyree and Sperry 1988). "Run-away cavitation," which is the loss of all hydraulic conductivity caused by unregulated ψ_P, can be induced experimentally, and it is a dramatic and quick cause of mortality (Holtta et al. 2012). Not surprisingly, plants have evolved the necessary cavitation resistance and stomatal control mechanisms to avoid such an efficient suicidal scenario. But stomatal control cannot directly prevent the gradual accumulation of cavitation as the xylem ψ_P becomes more negative due to limited soil water availability. In consequence, extreme stress events or climatic shifts push plants towards excessive cavitation, resulting in partial or complete dieback from chronic reductions (70% or greater) in hydraulic conductivity from cavitation (Anderegg et al. 2012; Plaut et al. 2012). Soil-plant-atmosphere models that incorporate cavitation resistance can be successful in predicting responses of vegetation to drought (Sperry et al. 2002), allowing effects of climate change on plant water and carbon flux to be anticipated.

Refilling of embolized conduits

The refilling of embolized xylem conduits has been documented in numerous studies. Embolisms accumulating over the winter from freeze-thaw cycles and other causes can be reversed in the spring in many species (Sperry 1993; Hacke and Sauter 1996). Diurnal embolism and refilling cycles have also been documented (Stiller et al. 2005; Yang et al. 2012), as well as refilling after relief from prolonged drought (West et al. 2008).

Two kinds of refilling have been observed. In "bulk" refilling, the sap ψ_P of the entire bulk xylem stream rises close to or above zero to force sap back into the embolized conduits. For this to happen, at a minimum, transpiration must be negligible and the soil ψ_w must be close to zero. Xylem ψ_P would thus decrease only to what is necessary to counter-act gravity, dropping by approximately 0.01 MPa per meter height. Less negative ψ_P values, even positive values, could develop from foliar uptake of rain or dew, and especially from osmotically

generated root pressures. A few species, *Acer* in particular, also develop positive stem ψ_P values in response to freeze-thaw cycles in early spring (Tyree and Zimmermann 2002). Root pressures can exceed 0.5 MPa and are strongly associated with bulk refilling in a variety of woody and herbaceous plants (Sperry 1993; Stiller et al. 2005; Yang et al. 2012). Experimental suppression of root pressure has been shown to block refilling in some species (Sperry 1993). The natural failure of root pressure and spring refilling, owing to freezing-related mortality of shallow roots, has been linked to birch dieback episodes (Cox and Malcolm 1997). Diminishing root pressure with plant height has also been invoked as a limit to the stature of refilling bamboos (Yang et al. 2012).

In a second type of "novel refilling," the bulk xylem ψ_P is much too negative to allow sap to move back into the embolized conduits (Salleo et al. 1996). There must be a pumping mechanism that brings sap into the embolized conduit and keeps it there until the gas is dissolved or escapes. The pumping mechanism is unknown, but several hypotheses have been proposed (see Nardini et al. 2011a for a recent review). Two basic driving forces are suggested: forward osmosis associated with solute accumulation in the thin water film along the embolized conduit wall, or reverse osmosis driven by either tissue pressure, or perhaps more likely, Münch pressure flow redirected from the phloem to the embolism, via ray tissue. In the latter case, refilling becomes a special case of phloem unloading. The data suggest the mechanism is: (a) triggered by the presence of a gas-filled conduit (rather than a particular plant water potential (Salleo et al. 1996)), (b) associated with starch hydrolysis (Bucci et al. 2003; Salleo et al. 2009), (c) upregulation of certain aquaporins (Secchi and Zwieniecki 2010), and (d) active phloem transport in the vicinity of the embolism (Salleo et al. 2006).

Xylem conduit wall sculpturing and chemistry may also be important (Kohonen and Helland 2009) with wettable areas assisting water uptake and gas dissolution, and hydrophobic areas perhaps allowing gas escape through minute wall pores (Zwieniecki and Holbrook 2009). Two very different roles have been proposed for inter-conduit pits in the refilling process. In one model, air pockets in the pit chamber and "wicking" forces at the aperture serve to isolate the pressurized sap in the embolized vessel from the negative ψ_P in the adjacent transpiration stream (Zwieniecki and Holbrook 2000). Alternatively, it has been proposed that pit membranes can act as osmotic membranes, with sap being pulled from the transpiration stream into the refilling conduit by an osmotic gradient, analogous to the generation of positive turgor pressures in protoplasts (Hacke and Sperry 2003).

A particularly informative study is the imaging work of Brodersen et al. (2010). Embolized vessels in grapevine were observed to refill while the ψ_P of the surrounding xylem was more negative than -0.7 MPa, confirming the need for a pumping process. Water entered empty vessels from the direction of the rays, a pattern consistent with phloem-directed water influx rather than either pit membrane osmosis from the transpiration stream or root pressure. There was no obvious mechanism to prevent drainage of the accumulating water back into the surrounding sap stream, contradicting a role of inter-vessel pits in hydraulic isolation. Whether the vessel refilled or stayed partially embolized depended on the difference between the rate of water influx from the rays, *versus* drainage to the surrounding transpiration stream. There was considerable variation in the onset, rate, and eventual success or failure, in the plants imaged. Unfortunately, the ψ_π of the refilling sap was not determined, so forward- versus reverse-osmosis mechanisms could not be distinguished. Nevertheless, the results lend strong support to a phloem-coupled refilling mechanism that refills by pumping water into the embolized vessels faster than it is withdrawn.

Engineering xylem properties: A path to increased plant productivity

The cohesion-tension mechanism constrains the productivity and survival of plants, arguably constituting the "functional backbone of terrestrial plant productivity" (Brodribb 2009). Because of the stomatal regulation of canopy xylem ψ_P, frictional resistance to water flow through the plant is coupled to the maximum potential photosynthetic rate and, hence, to productivity in general. The coupling in turn is necessary for avoiding hydraulic failure by cavitation, which limits plant survival *in extremis*. The cavitation limit presumably evolved in response to complex trade-offs with frictional resistance, with competition selecting for minimal flow resistance at the expense of excessive cavitation safety margins. Although the driving force for the transpiration stream is passive, flow resistance (via the ionic effect) and conduit refilling is modulated by active metabolic processes. Probably the single most important structures in the pipeline are the inter-conduit pits: their distribution, chemistry, structure, and mechanical properties greatly influence both frictional resistance to flow and vulnerability to cavitation by water stress.

The tools of molecular biology have the potential to greatly advance our knowledge of the flow resistance, cavitation, and refilling phenotypes, as well as the nature of trade-offs among them. As the genetic and developmental controls of xylem anatomical traits become better understood (Demari-Weissler et al. 2009), they can be manipulated to untangle structure-function relationships that can otherwise only be inferred from comparative studies. Crucial to advancement in this area are model organisms in which the hydraulic physiology can be phenotyped and manipulated. Among woody plants, the *Populus* system is perhaps most promising, and much has already been learned from it (Secchi and Zwieniecki

2010; Schreiber et al. 2011). The next decade should bring rapid progress as molecular biology continues to merge with comparative and evolutionary whole plant physiology.

Long-distance Signaling Through the Phloem

Over the past several decades, considerable attention has been paid to unraveling the mechanics of phloem loading. Genetic and molecular studies have identified the major players that mediate in the loading of sugars, predominantly sucrose, into the CC-SE complex. Interestingly, in terms of the apoplasmic loaders, the recent identification of the permease that controls release of sucrose from the phloem parenchyma cells into the CC apoplasm (**Figure 13B, I**) served to complete the molecular characterization of this important pathway (Chen et al. 2012b). Based on such studies and extensive physiological experiments, the nature of the photosynthates (sugars and amino acids) loaded into the phloem translocation stream is well established.

The phloem has also been shown to carry additional cargo, including the phytohormones auxin, gibberellins, cytokines and abscisic acid (Hoad 1995), signaling agents involved in plant defense (discussed later in the review), as well as certain proteins and various forms of RNA (Lough and Lucas 2006; Buhtz et al. 2010). That specific proteins are present in the phloem has been recognized for some time (Fisher et al. 1992; Bostwick et al. 1992), and furthermore, some such proteins have been shown to move within the translocation stream (Golecki et al. 1998, 1999; Xoconostle-Cázares et al. 1999).

Phloem proteins: Potential roles in enucleate SE maintenance and long-distance signaling

Phloem exudate can be collected from a number of plant species, and this feature has been used to develop proteomic databases for these species (Barnes et al. 2004; Giavalisco et al. 2006; Lin et al. 2009; Rodriguez-Medina et al. 2011). This collection process requires that an incision be made into the petiole or stem in order to allow the phloem to "bleed." Thus, due care is required to minimize the level of protein contamination from surrounding (CCs and phloem parenchyma) tissues. As excision results in an abrupt pressure drop between the sieve tube system and the surrounding cells, it is generally appreciated that some level of contamination is unavoidable (Atkins et al. 2011). Here, use of molecular markers such as Rubisco (Doering-Saad et al. 2006; Giavalisco et al. 2006; Lin et al. 2009), can help in assessing the extent to which contamination may have occurred. Generally, contamination does not appear to be an important issue, especially for the prominent proteins, but proteins present in very low abundance need to be viewed with a degree of caution.

Other methods, including cutting aphid stylets (Aki et al. 2008; Gaupels et al. 2008a), EDTA-induced phloem exudation (Gaupels et al. 2008b; Batailler et al. 2012) and laser microdissection of phloem tissues (Deeken et al. 2008) have also been employed to develop phloem databases. Collectively, these studies have established phloem proteome databases containing more than 1,000 proteins, with activities encompassing a very broad range of activities, including enzymes involved in metabolic networks, amino acid synthesis, protein turnover, RNA binding, transcriptional regulation, stress responses, defense, and more.

The next step will be to partition these proteins into those involved in local maintenance of the functional enucleate sieve tube system and long-distance signaling. For these studies, a combination of hetero-grafting experiments conducted between species from different genera or families, and advanced mass spectroscopy methods, will prove most useful. The cucurbits, such as pumpkin, cucumber, melon and watermelon, from which analytical quantities of phloem exudate can generally be collected, may prove ideal for this purpose. The recent completion of annotated genomes for three of these cucurbits (Huang et al. 2009; Garcia-Mas et al. 2012; Guo et al. 2012) adds to the utility of these species for such critical experiments.

The complexity of the phloem proteome raises the question as to the stability of these proteins and the mechanism by which they might be turned over within the sieve tube system. The large population of proteinase inhibitors probably prevents turnover by simple proteolysis (Dinant and Lucas 2012). However, identification in the phloem sap of ubiquitin and numerous enzymes involved in protein ubiquitination and turnover, including all the components of the 26S proteasome (**Figure 19**), indicates that enucleate SEs likely have retained the ability to engage in protein sorting and turnover (Lin et al. 2009). Thus, once they have performed their function(s), phloem proteins can be degraded either through export into neighboring CCs, or *in loco* via the ubiquitin-26S proteasome pathway.

The mature, enucleate sieve tube system also has been shown to contain all the enzymes and associated activities required for a complete antioxidant defense system (Walz et al. 2002; Lin et al. 2009; Batailler et al. 2012). Interestingly, these enzyme activities appear to increase in response to imposed drought stress (Walz et al. 2002). This complement of enzymes would appear to function, locally, to afford protection against oxidative stresses, thereby preventing damage to essential components of the SEs. Such local maintenance functions will likely be performed by a specific subset of the proteins detected in phloem exudates.

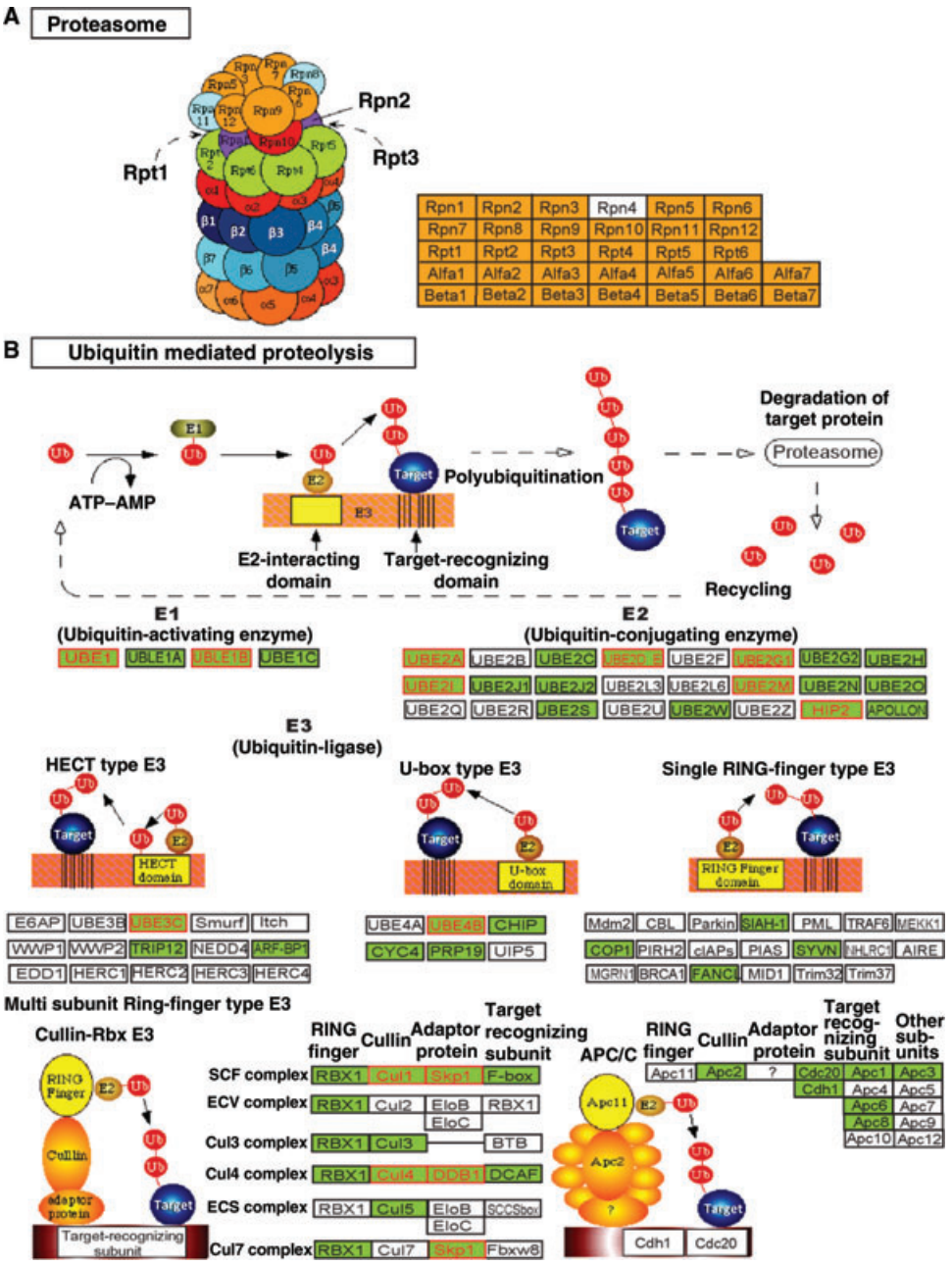

Figure 19. Pumpkin phloem sap contains the machinery for ubiquitin-mediated proteolysis.

(A) Schematic representation of the 26S proteasome indicating that all components except for Rpn4 were identified from the pumpkin phloem sap. Orange boxes indicate components identified by Lin et al. (2009).

(B) Identification of phloem proteins associated with ubiquitin-mediated proteolysis. Note that green boxes represent proteins present in the *Arabidopsis* genome, and red lettering indicates identification in pumpkin phloem proteins. White boxes represent *Saccharomyces cerevisiae*-specific proteins. Ub, ubiquitin; CHIP, carboxyl terminus of Hsc 70-interacting protein; APC/C, anaphase promoting complex/cyclosome; DCAF, DD B1-CUL4-associated factor; SCF, Skp1-Cul1-F-box protein; ECS, Elongin C-Cul2-SOCS box; ECV, SCF-like E3 ubiquitin ligase complex (from Lin et al. 2009).

FLOWERING LOCUS T (FT) as the phloem-mobile florigenic signal

It has long been known that, for plants whose flowering is controlled by day length, the phloem is involved in the transmission of a photoperiod-induced signal that moves from the mature/source leaves to the shoot apex where it induces the onset of flowering (Zeevaart 1976). The nature of this graft-transmissible signal, termed florigen (Zeevaart 2006), was recently identified as FT, a member of the CETS protein family (consisting of CENTRORADIALIS [CEN], TERMINAL FLOWER 1 [TFL1] and FT). *FT* expression is confined to CCs in source leaves, and this small protein enters the sieve tube system by passage through the CC-SE PD.

Direct evidence for the presence of FT in the phloem translocation stream was provided by studies performed on a pumpkin (*Cucurbita moschata*) accession in which flowering occurs only under short-day (SD) conditions (Lin et al. 2007). Mass spectroscopy studies conducted on phloem sap collected from plants grown under long-day (LD) and SD conditions provided unequivocal evidence that the *C. moschata* FT orthologue was present only in exudate collected from SD-grown plants in which flowering was induced. Supporting evidence for FT as a component of the long-distance florigenic signal was provided by studies on *Arabidopsis* (Corbesier et al. 2007) and rice (Tamaki et al. 2007). Here, *FT* and *Hd3a*, the rice *FT* orthologue, were expressed as *GFP* fusions driven by a CC-specific promoter. Detection of a GFP signal in the meristem of these transgenic plants was consistent with movement from the phloem into the meristem where floral induction was taking place.

An absolute quantitation peptide approach was used to determine the level of FT in the pumpkin phloem translocation stream. Recorded values were in the low femtomolar range (Lin et al. 2007), clearly placing FT in a protein hormone category (Shalit et al. 2009). Here, it is noteworthy that FT peptides could not be detected in these pumpkin phloem exudates when analyzed using the proteomics approach reported by Lin et al. (2009). This is important, as it indicates that not all low abundance proteins detected in phloem exudates represent contaminants from surrounding tissues.

As a number of examples exist in which both mRNA and the encoded protein have been detected in phloem exudates (Lough and Lucas 2006), Lin et al. (2007) carried out extensive tests for the presence of the pumpkin *FT* transcripts using the same phloem exudates in which FT peptides were identified. As *FT* transcripts could not be amplified, it appeared that, in these plants, FT protein, but not its mRNA, serves as the florigenic signal. This finding was consistent with earlier studies conducted on the tomato *FT* orthologue, *SINGLE-FLOWER TRUSS* (*SFT*). SFT-dependent graft-transmissible signals were found to induce flowering in day length neutral tomato and tobacco plants; however, no evidence was obtained for the presence of *SFT* transcripts within the scion meristem tissues (Lifschitz et al. 2006). Extensive mutagenesis of the *FT* gene, in which sequence and structural modifications were made to the mRNA whilst leaving the FT protein unaltered, had little or no effect on long-distance floral induction (Notaguchi et al. 2008). Again, these findings are consistent with FT protein, not its mRNA serving as the phloem-mobile signal.

Experimental support for *FT* mRNA as a component of the florigenic signaling mechanism has been suggested from studies based on movement-defective viral expression systems (Li et al. 2009). Here, the first 100 nucleotides of the *FT* RNA sequence were shown to function in *cis* to allow systemic movement of heterologous viral sequences. Similar findings with *Arabidopsis* have been reported in terms of *FT* sequences acting in *cis* to mediate in the long-distance transport of otherwise cell-autonomous transcripts (Lu et al. 2012). Further support for the role of endogenous *FT* mRNA, as a component of the florigenic signal, was claimed from studies with transgenic *Arabidopsis* lines in which flowering was delayed through expression, in the apex, of RNAi/artificial *miRNA-FT* (Lu et al. 2012). Unfortunately, these results are contradictory to findings from a similar *FT* silencing experiment in which expression of *amiRNA-FT* in the phloem caused a significant delay in floral induction, whereas its expression in the apex failed to delay flowering (Mathieu et al. 2007). Although the controversy over whether or not *FT* mRNA contributes to floral induction remains to be resolved, there is no *a priori* reason why, for any gene, its mRNA and protein cannot both serve as signaling agents via the phloem.

ATC as a phloem-mobile anti-florigenic signal

Systemic floral inhibitors or anti-florigens have been proposed to participate in down regulating floral induction under non-inducing conditions (Zeevaart 2006). Evidence in support of this concept was recently provided by studies performed on *Arabidopsis* plants carrying mutations in *ATC*, a *CEN*/*TFL1* homologue. Flowering in *Arabidopsis* is promoted under LD- and inhibited under SD-conditions. Expression of *ATC* is enhanced during short days and, based on the effect of an *atc-2* mutant, the WT gene appears to contribute to the suppression of flowering (Huang et al. 2012).

At the tissue level, *ATC* was found to be expressed in the vascular system, and the phloem in particular, but not in the apex. This suggested a non-cell-autonomous function for either the *ATC* transcripts or protein. A range of grafting experiments were performed with *Arabidopsis* stock and scions connected above the hypocotyl region (with the stock containing several mature source leaves). Analysis of RNA extracted from *atc-2*

scions grafted onto WT stocks provided clear evidence for the movement of *ATC* transcripts across the graft union, and compared to WT:WT grafts, flowering time was delayed (Huang et al. 2012). Parallel experiments were performed to address whether ATC protein moves across the graft union. In these Western blot experiments, a clear ATC signal was detected in *atc-2* scions grafted onto WT stocks. These findings support the possibility that, in *Arabidopsis*, both *ATC* transcripts and protein are phloem-mobile; i.e., together, they may enter the shoot apex to compete with FT for FD, thereby inhibiting the transition to flowering. However, it is also possible that the phloem-mobile *ATC* transcripts enter CCs located in the *atc-2* scion tissues where they then produce ATC protein. Irrespective of this potential complication, identification of ATC as a negative regulator of flowering time in *Arabidopsis* constitutes an important step forward in understanding the role of the phloem in the overall regulation of plant growth and development.

Phloem-mediated long-distance lipid-based signaling?

Lipids and lipid-binding proteins have been detected in phloem exudates collected from a number of plant species. Some 14 putative lipid-binding proteins were detected in *Arabidopsis* phloem exudates collected from excised petioles that were incubated in EDTA to facilitate the bleeding process (Guelette et al. 2012). Bioinformatics analysis of these proteins indicated potential roles in membrane synthesis and/or turnover, prevention of lipid aggregation, participation in synthesis of the glycosyphosphatidylinositol (GPI) anchor, and biotic and abiotic stress. A range of lipids have also been reported in phloem exudates, including simple lipids to complex glycolipids and phytosterols such as cholesterol (Behmer et al. 2011; Guelette et al. 2012).

An interesting study recently conducted on an *Arabidopsis* small (20 kDa) phloem lipid-associated family protein (PLAFP) revealed that it displayed specific bind properties for phosphatidic acid (PA) (Benning et al. 2012). As both PA and PLAFP were detected in *Arabidopsis* exudate, these results suggest that PA may well be either trafficked into or translocated through the sieve tube system by PLAFP. In any event, detection of lipids and lipid-binding proteins within phloem exudates certainly raises the question as to whether they function in membrane maintenance and/or long-distance signaling events.

Messenger RNA: A smart way to send a "message"!

A number of recent studies have identified specific mRNA populations within the phloem sap of various plant species (Sasaki et al. 1998; Doering-Saad et al. 2006; Lough and Lucas 2006; Omid et al. 2007; Deeken et al. 2008; Gaupels et al. 2008a; Rodriguez-Medina et al. 2011; Guo et al. 2012). These databases indicate that the phloem translocation stream of the angiosperms likely contains in excess of 1,000 mRNA species that encode for proteins involved in a very wide range of processes. While many of these transcripts are held in common between plant species, specific differences have been reported. For example, a comprehensive analysis carried out using the phloem transcriptomes prepared from cucumber (1,012 transcripts) and watermelon (1,519 transcripts) phloem exudate indicated that 55% were held in common (Guo et al. 2012). In contrast, the vascular transcriptomes (13,775 and 14,242 mRNA species in watermelon and cucumber, respectively) were 97% identical. Thus, differences in phloem transcripts most likely reflect unique functions specific to these species.

A comparative analysis of the vascular and phloem transcriptomes for cucumber and watermelon identified populations of transcripts that are highly enriched in phloem exudates over the level detected in excised vascular bundles. The numbers given above represent the transcripts that were present at $\geq$2-fold higher than the level detected in vascular bundles. Concerning cucumber, more than 30% of the phloem transcripts were enriched $>$10-fold above the level in the vascular bundles. Importantly, some transcripts were enriched above 500-fold, with another 210 displaying $>$20-fold enrichment. A similar situation was observed for watermelon, with some 120 transcripts displaying $>$10-fold enrichment and 320 having 5-fold or greater enrichment in the phloem sap. These data indicate that, following transcription in the CCs, many transcripts must undergo sequestration in the sieve tube system through trafficking mediated by the CC-SE PD.

To date, only a limited number of these phloem mRNAs have been characterized in terms of whether they act locally or traffic long-distance to specific target sites. Excellent examples where translocation through the phloem has been established include *NACP* (Ruiz-Medrano et al. 1999), *PP16* (Xoconostle-Cázares et al. 1999), the *PFP-LeT6* fusion gene (Kim et al. 2001), *GAIP* (Haywood et al. 2005), *BEL5* (Benerjee et al. 2006; Hannapel 2010), *POTH1* (a KNOTTED1-Like transcription factor) (Mahajan et al. 2012) and *Aux*/*IAA18* and *Aux*/*IAA28* (Notaguchi et al. 2012). The stability of these phloem-mobile transcripts is made possible by the fact that phloem exudates have been shown to lack RNase activity (Xoconostle-Cázares et al. 1999), and thus, by extension, the phloem translocation stream is likely also devoid of this activity.

Phloem delivery of *GAIP* transcripts modifies development in tomato sink organs

The pumpkin phloem sap was found to contain transcripts for two members of the DELLA subfamily of GRAS transcription factors, *CmGAIP* and *CmGAIPB*, known to function in the GA signaling pathway (Ruiz-Medrano et al. 1999). The

function of *CmGAIP* and *GAI* from *Arabidopsis* was investigated using transgenic *Arabidopsis* and tomato lines expressing engineered dominant gain-of-function *Cmgaip* and *ΔDELLA-gai* genes. Importantly, these transgenic plants exhibit clear morphological changes in leaf development, and this characteristic was used to test whether phloem delivery of the *Cmgaip*/*ΔDELLA-gai* transcripts into sink tissues could induce this mutant phenotype. These engineered *gai* transcripts were found to move long-distance through the phloem, and could then exit from the terminal phloem and subsequently traffic into the apex, where they accumulated in developing leaf primordial (Haywood et al. 2005). Parallel studies conducted with transgenic plants expressing *EGFP* revealed the inability of these transcripts to enter the phloem. This finding suggested that phloem entry of *Cmgaip*/*ΔDELLA-gai* transcripts must occur by a selective process.

Analysis of WT tomato scions grafted onto *Cmgaip* and *ΔDELLA-gai* stocks indicated that import of these transcripts caused highly reproducible morphological changes in leaf phenotype (**Figure 20**). Unexpectedly, tomato leaflet morphology was found to be influenced quite late in development. Of equal importance, the presence of *Cmgaip* and *ΔDELLA-gai* transcripts, within the various tissues of the scion, was not correlated with overall sink strength. Strong signals were detected in young developing flowers and the apex, but signal could not be amplified from fruit stalks or rapidly expanding fruit (**Figure 20C**). These findings indicated an unexpected complexity in the events underlying phloem delivery of these transcripts, suggesting a high degree of regulation over such trafficking of macromolecules. Furthermore, these studies revealed that phloem long-distance delivery of RNA can afford flexibility in adjusting developmental programs to ensure that newly emerging leaves are optimized for performance under existing environmental conditions (Haywood et al. 2005).

A model has been proposed that selective entry of transcripts from the CC into the sieve tube system involves specific sequences within the RNA (Lucas et al. 2001). As both *Cmgaip* and *ΔDELLA-gai* transcripts were able to move within the heterologous plant, tomato, this finding suggested that such sequence motifs, termed "zip codes," must be conserved and, further, the molecular machinery required for this recognition and trafficking must similarly be conserved between pumpkin, tomato and *Arabidopsis.*

Support for this hypothesis was provided by mutational analysis of *GAI* in which it was clearly established that mRNA entry into the phloem is facilitated by a motif located within the 3′ region of the transcript (Huang and Yu 2009). Furthermore, this motif was specific to *GAI*, as parallel experiments conducted with the four additional members of the DELLA family failed to detect their transcripts in heterografting assays. By testing an extensive series of *GAI* mutants, it was found that two zip

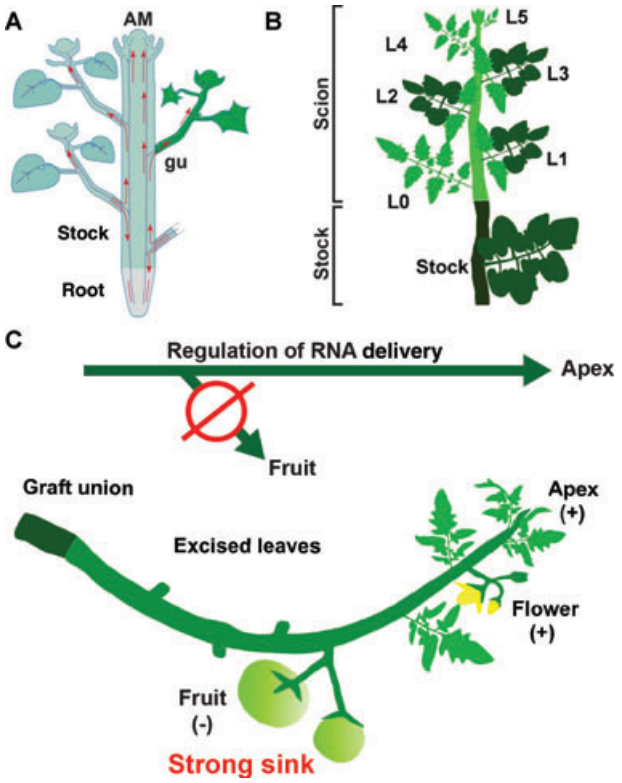

Figure 20. Phloem delivery of *Cmgaip*/*ΔDELLA-gai* transcripts modifies leaf development.

(A) Schematic illustrating the V-grafting method used to test the influence of phloem-mobile transcripts, derived from the stock and imported across the graft union (gu) into the scion, on developmental processes occurring in the scion.

(B) Schematic showing that import of *Cmgaip*/*ΔDELLA-gai* transcripts into wild-type tomato scions results in the development of scion leaves with characteristic morphological features associated with transgenic stock plants carrying the dominant gain-of-function *Cmgaip* or *ΔDELLA-gai* gene (Haywood et al. 2005). Numbers represent tomato leaflet position along the leaf axis. Leaflet L0 does not exhibit this change in morphology as it had passed through this developmental stage prior to formation of the graft union.

(C) Detection of *Cmgaip*/*ΔDELLA-gai* transcripts in wild-type tomato scions grafted onto *Cmgaip*/*ΔDELLA-gai* transgenic tomato stocks; presence (+), absence (-). Absence of phloem-mobile transcripts from the rapidly growing fruit indicates the operation of a regulatory system controlling delivery of phloem mobile transcripts to specific tissues/organs (from Haywood et al. 2005).

codes appear to be required for phloem entry and translocation, one being located in the coding sequence and the other in the 3′ untranslated region. Finally, *GFP* transcripts carrying these two zip codes were detected in the scion, confirming that these sequence motifs are necessary and sufficient for targeting *GAI* transcripts to the phloem (Huang and Yu 2009).

Role of phloem RNP complexes in RNA delivery to target tissues

Considering that the phloem translocation stream contains in excess of 1,000 transcripts, it is perhaps not surprising that the pumpkin phloem proteome was found to contain in excess of 80 recognized RNA binding proteins (RBPs) (Lin et al. 2009). Several of these RBPs have been characterized, with the first being CmPP16-1 and CmPP16-2 from pumpkin (Xoconostle-Cázares et al. 1999). These two proteins display properties equivalent to those of viral movement proteins (Lucas 2006) in that they bind RNA in a sequence non-specific manner and mediate the cell-to-cell trafficking of transcripts through PD.

Entry of CmPP16-1/2 into the sieve tube system appears to be controlled by post-translational modifications. Interestingly, both CmPP16 and its PD receptor, NCAPP1 (Lee et al. 2003), require serine residues to be phosphorylated and glycosylated for effective interaction and delivery of CmPP16 to and through PD (Taoka et al. 2007). In an elegant experiment, Aoki et al. (2005) used severed brown leafhopper stylets to introduce CmPP16-1 and CmPP16-2 directly into the sieve tube system of rice. Analysis of the long-distance movement of these two pumpkin proteins, within the rice plant, clearly revealed that they did not simply follow the direction of bulk flow. Destination-selective movement was shown to be controlled by proteins from the pumpkin phloem sap that interact with CmPP16-1/2. Collectively, these studies on CmPP16 provide important insights into the complexity of the processes that underlie macromolecular trafficking within the phloem translocation system.

The most extensively characterized phloem RBP is CmRBP50, a polypyrimidine tract-binding protein that accumulates to high levels in pumpkin phloem sap (Ham et al. 2009). Pull down assays, using a polyclonal antibody directed against CmRBP50 and pumpkin phloem exudates, led to the identification of the proteins and mRNA species contained within a CmRBP50-associated ribonucleoprotein (RNP) complex (**Figure 21A**). Interestingly, *CmGAIP* transcripts were contained within this CmRBP50 RNP complex. Binding specificity between CmRBP50 and these *CmGAIP* transcripts is imparted by a series of polypyrimidine tracts located within the mRNA. As these sites differ from those involved in mediating CmGAIP transcript entry into the phloem (Huang and Yu 2009), it is likely that assembly of the CmRBP50 RNP complex occurs within the sieve tube system.

Heterografting studies conducted between pumpkin (stock) and cucumber (scion) established that this RNP complex is engaged in the long-distance delivery of *CmGAIP* mRNA to developing tissues. Important insights into the basis for the stability of this CmRBP50-*CmGAIP* mRNA complex were provided by reconstitution experiments. These studies identified a series of serine residues within the CmRBP50 C-terminus that, when phosphoryated, allow for the assembly of the RNP complex. Sequential binding of CmPP16 and the other proteins that form the complex results in an increase in its overall stability (Li et al. 2011) (**Figure 21B**).

In addition to *CmGAIP*, *CmSCARECROW-LIKE*, *CmSHOOT MERISTEMLESS, CmETHYLENE RESPONSE FACTOR* and *CmMybP* transcripts were also isolated from CmRBP50 RNP complexes. Given that the watermelon phloem exudate was found to contain transcripts for some 118 transcription factors, there remains much to be done in terms of identifying and characterizing the associated RNP complexes involved in mediating their entry into, and presumed long-distance transport through, the phloem.

Phloem transcripts and protein synthesis in the enucleate sieve tube system

Analysis of cucumber phloem proteome and transcriptome databases identified some 169 proteins for which transcripts were also present in phloem exudates. This represents around 15% of the phloem transcripts and raises the question as to why there would be the need for such transcripts when, presumably, the proteins can enter the sieve tube system by trafficking through CC-SE PD. The possibility exists that some proteins required for SE maintenance are cell-autonomous. If this were the case, synthesis within the enucleate sieve tube system would be required. It has long been assumed that the mature SE does not have the capacity for protein synthesis. However, the pumpkin phloem proteome contains numerous proteins involved in translation (Lin et al. 2009). Furthermore, gel filtration chromatography experiments performed on pumpkin phloem exudates identified complexes of proteins containing CmeIF5A and elongation factor 2, both known to be involved in protein synthesis (Ma et al. 2010). Thus, synthesis of a discrete set of essential proteins may well occur within mature SEs.

Phloem-based delivery of small RNA and systemic gene silencing

In recent years, post-transcriptional gene silencing has emerged as an important component of the regulatory networks that control a broad array of developmental and physiological processes (Brodersen and Voinnet 2006). These events can occur in local tissues, and the phloem also functions as a conduit for the systemic spread of gene silencing (Melnyk et al. 2011). The pioneering work of Palauqui and coworkers laid a solid foundation for this concept. Transgenic tobacco plants expressing additional copies of a nitrate reducase gene (*Nia*) were found to undergo a perplexing process in which small clusters of cells within mature source leaves were observed to

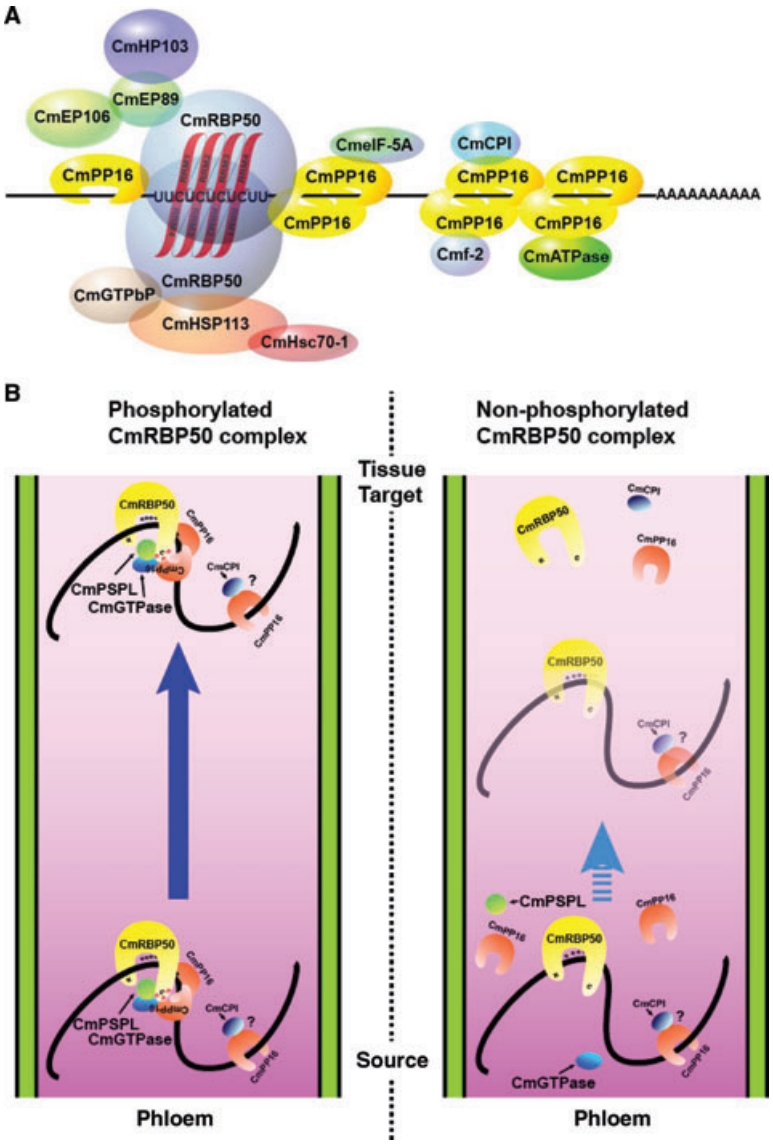

Figure 21. Delivery of phloem-mobile transcripts to sink tissues requires formation of stable ribonucleoprotein complexes.

(A) Model illustrating the protein composition of the pumpkin CmRBP50-based ribonucleoprotein (RNP) complex that binds specifically to five phloem transcripts that encode transcription factors which are delivered into sink tissues (from Ham et al. 2009).
(B) Phosphorylation of four serine residues at the C-terminus of CmRBP50 is essential for assembly of a stabilized RNP complex (left image). Mutating these serine residues prevents RNP complex assembly in the sieve tube system (right image) (from Li et al. 2011).

turn white (Palauqui et al. 1996). Subsequently, this process moved up the body of the plant in a source-to-sink pattern reflective of phloem translocation.

An insightful follow-up series of experiments revealed that a graft-transmissible signal moved into the scion where it caused the turnover of *Nia* transcripts. A deficiency in fixed nitrogen then caused the leaves of the scion to turn white (Palauqui et al. 1997) (**Figure 22A**). Cell-to-cell movement of the *Nia* silencing signal was tested by placement of a WT stem segment between the silenced stock and the non-silenced scion. That white leaves still developed in these scions confirmed the involvement of the phloem (**Figure 22B**). This conclusion was further supported by grafting *Nia*-silenced scions onto non-silenced root stocks. As the direction of the phloem is from the stock to the scion, this graft combination did not result in the generation of white leaves (**Figure 22C**), again consistent with transmission of the silencing signal through the phloem.

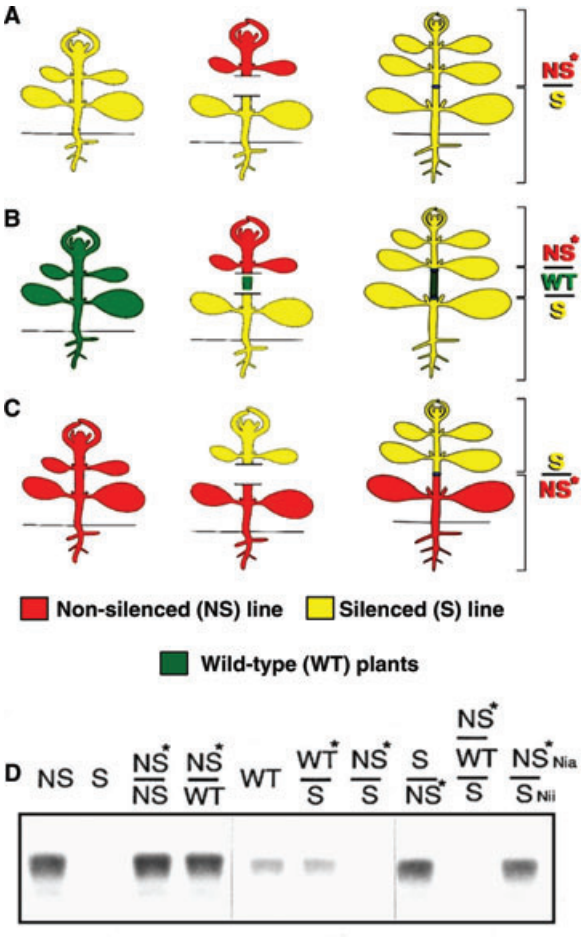

Figure 22. Grafting experiments illustrating that transmission of a phloem long-distance signal can induce post-transcriptional gene silencing within developing scion tissues.

(A–C) Transgenic tobacco plants expressing a nitrate reductase gene (*Nia*) segregated into silenced (S) and non-silenced (NS) phenotypes were employed in grafting studies to test for the long-distance propagation of the silencing condition through the phloem. **(A)** Grafting of an NS scion onto an S stock resulted in silencing of *Nia* within the scion leaves. **(B)** Placement of a wild-type (WT) stem segment between the NS scion and S stock did not prevent the transmission of the silencing signal. **(C)** Grafting of a silenced scion (S) onto a NS stock does not activate silencing in the NS stock tissues.

(D) Analysis of RNA samples collected from specific grafted tissues (*) confirmed that the observed silencing phenotype reflected sequence-specific targeting of the *Nia* transcripts (redrawn from Palauqui et al. 1997).

Sequence-specificity of the silencing signal was established by grafting studies performed with nitrite reductase (*Nii*) transgenic tobacco stocks and non-silenced *Nia* scions (**Figure 22D**). A hypothesis was advanced that phloem-mobile silencing signals involved the translocation of antisense RNA, whose entry into the developing scion tissues caused an enzyme-mediated cleavage of the double-stranded form of the target RNA (Jorgensen et al. 1998). Detection, in silenced tissues, of small (20–25 nucleotide [nt]) antisense RNA complementary to the silenced gene (sRNA) (Hamilton and Baulcombe 1999) provided strong support for the general features of this model.

Analysis of RNA extracted from pumpkin phloem sap identified a population of 21 nt – 24 nt sRNA. Sequencing and bioinformatics analysis indicated that these sRNAs belong to both the micro(mi)RNA and small interfering (si)RNA silencing pathways (Yoo et al. 2004). Interestingly, although equal signal strength was detected for sense and antisense sRNA probes, they did not appear to exist in the phloem sap as duplexes. The involvement of these phloem sRNAs in systemic silencing was explored using silencing (stock) and non-silencing (scion) transgenic squash (*Cucurbita pepo*) plants expressing a viral coat protein gene. Phloem sap from both the stock and scion tested positive for *CP* siRNA, and analysis of apical tissues from these scions confirmed that the level of *CP* mRNA had been greatly reduced. Interestingly, a low level signal for the antisense *CP* transcript could also be detected in the phloem sap of both stock and scion plants. Collectively, these findings offered support for the hypothesis that both siRNA and antisense RNA are likely components of the systemic silencing machinery.

Limited information is available concerning the mechanism by which these sRNA molecules enter and move long-distance through the phloem. Biochemical studies performed on pumpkin and cucumber phloem exudate identified a 20 kDa PHLOEM SMALL RNA BINDING PROTEIN1 (PSRP1) that bound specifically to sRNA (Yoo et al. 2004). This protein has the capacity to traffic its sRNA cargo through PD, and in the cucurbits, PSRP1 may be involved in shuttling sRNA from CCs into the sieve tube system. Interestingly, PSRP1 homologues have yet to be identified in the genomes of other plant species. This raises the possibility that additional proteins have evolved to carry out these same functions.

Role of phloem-mobile sRNA in directing transcriptional gene silencing in target tissues

The phloem sap collected from pumpkin and oilseed rape contains a significant population of 24-nt sRNA (Yoo et al. 2004; Buhtz et al. 2008), indicating a likely involvement in transcriptional gene silencing (TGS) within sink tissues (Mosher et al. 2008). Grafting experiments performed with various combinations of *GFP* transgenic and *DICER-LIKE* mutant *Arabidopsis* lines provided further confirmation that a significant population of exogenous/endogenous 23 nt – 24 nt sRNA can cross the graft union (Molnar et al. 2010). Methylation analysis of DNA extracted from these grafted target tissues provided

compelling evidence for the hypothesis that phloem-mobile 24-nt sRNA can mediate in epigenetic TGS of specific genomic loci.

Similar findings were reported for studies on endogenous inverted repeats that generate double-stranded RNA molecules (Dunoyer et al. 2010), as well as for transgenic plants expressing a hairpin-structured gene under the control of a viral companion-cell-specific promoter (Bai et al. 2011). In this latter case, this phloem-transmissible TGS event was shown to be inherited by subsequent progeny. Collectively, these findings support the notion that phloem-mobile sRNA can serve to regulate gene expression within developing tissues epigenetically to allow for adaptation to environmental inputs.

Phloem-mobile miRNA

Although generally considered to act cell-autonomously (Voinnet 2009), as mentioned above, numerous miRNAs have been detected in phloem exudates from various plant species, and the roles played by some of these have recently been established. In plants, adaptation to changing nutrient availability in the soil involves both root-to-shoot (see next section) and shoot-to-root signaling. In the case of phosphate (Pi), changes in availability within leaves leads to an upregulation in miR399 production and, subsequently, its entry into the phloem translocation stream (Lin et al. 2008; Pant et al. 2008). Delivery of miR399 into the roots results in the cleavage of the target mRNA encoding for PHO2, a ubiquitin-conjugating E2 enzyme (UBC24). This gives rise to increased uptake of Pi into these roots and restoration of Pi levels within the body of the plant. Loss of PHO2 activity probably allows for an increase in Pi transporter capacity; i.e., of influx carriers located in the outer region of the root and xylem parenchyma-located efflux carriers that function in Pi loading into the transpiration stream (Chiou and Lin 2011).

Tuber induction in potato is regulated by phloem delivery of *BEL5* transcripts and miR172 (Martin et al. 2009). Vascular expression of *miR172* and its upregulation under tuber-inducing SD conditions suggested that this miRNA may act as a long-distance signaling component in the control of potato tuber induction. Support for this notion was provided by grafting studies involving P_{35S}*:MIR172* stocks grafted to WT potato scions. Here, tuberization occurred as early as in P_{35S}*:MIR172* potato lines. In contrast, when P_{35S}*:MIR172* scions were grafted to WT stocks, early tuber induction did not occur. Although these findings are consistent with *miR172* serving as a phloem-mobile signal, it is also possible that it might act through regulation, in the CCs, of an independent mobile signal.

Phloem-mobile sRNA control over host infection by parasitic plants

Parasitic plants cause major losses in some regions of the world (Ejeta 2007). Recent studies have established that host transcripts can enter into parasitic plants (Roney et al. 2007; David-Schwartz et al. 2008). The pathway for this trafficking is through the haustoria of the parasite that interconnects its vascular system to that of the host. This suggested that host invasion by parasitic plants might be controlled by phloem delivery of sRNA species designed specifically to target critical genes involved in the physiology or development of the parasitic weed (Yoder et al. 2009).

Based on the observation that parasitic broomrape (*Orobanche aegyptiaca*) accumulates large quantities of mannitol, Aly et al. (2009) engineered transgenic tomatoes to express a hairpin construct to target the mannose 6-phosphate reductase (*M6PR*) that functions as a key enzyme in mannitol biosynthesis. Analysis of tissue from broomrape growing on these transgenic tomato plants indicated a significant reduction in both *M6PR* transcript and mannitol levels. This strategy gave rise to a level of tomato protection against this plant parasite.

An alternate control approach based on targeting a parasitic developmental program involved the development of transgenic tobacco plants expressing hairpin constructs for two dodder (*Cuscuta pentagona*) haustoria-expressed *KNOTTED-like HOMEOBOX1* (*KNOX1*) genes. These constructs were driven by the CC-specific *SUC2* promoter and were based on 3′UTRs that did not display sequence homology to the related tobacco orthologues, STM and KNAT1–3 (Alakonya et al. 2012). Defects in haustoria development and connection to the transgenic tobacco plants were highly correlated with the presence of *KNOX1* siRNA, delivered most likely through the vascular system, and down-regulation of the *C. pentagona KNOX1* transcript levels. Importantly, dodder plants growing on these transgenic tobacco plants exhibited greatly reduced vigor. Collectively, these studies indicate that an effective control of plant parasitism may be achieved by targeting a pyramided combination of parasite genes involved in various aspects of growth and development.

Root-to-shoot Signaling

Response to abiotic stress

Signals arising within the root system can provide shoots with an early warning of root conditions, such as water deficiency, nutrient availability/deficiency, and so forth (**Figure 23**). The xylem transports hormones, such as abscisic acid (ABA) (Bahrun et al. 2002; Jiang and Hartung 2008), ethylene and cytokinin (CK) (Takei et al. 2002; Hirose et al. 2008; Kudo et al. 2010; Ghanem et al. 2011), as well as strigolactones (SLs) (Gomez-Roldan et al. 2008; Umehara et al. 2008; Brewer et al. 2013; Ruyter-Spira et al. 2012) from roots to aboveground tissues. In this section of the review, we will address the role of these xylem-borne signaling agents.

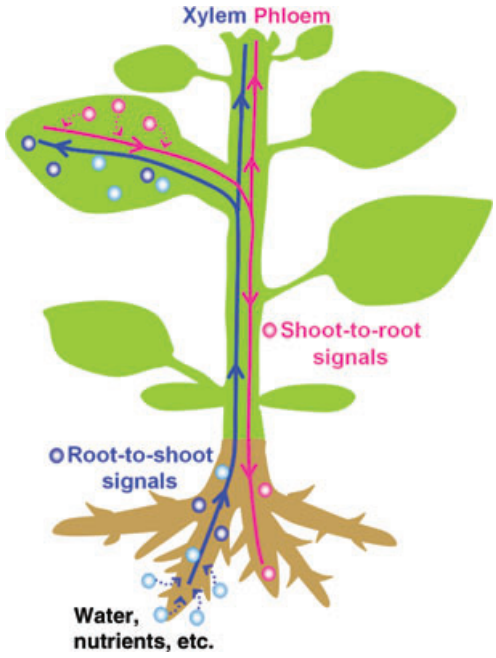

Figure 23. The plant vascular system serves as an effective inter-organ communication system.

In response to a wide range of environmental and endogenous inputs, the xylem (blue lines) transmits root-to-shoot signals (blue circles), including hormones such as abscisic acid, ACC (ethylene precursor) and cytokinin, as well as strigolactones (SLs). These xylem-borne signaling agents serve to communicate the prevailing conditions within the soil. The phloem (pink lines) transports a wide array of shoot-to-root signaling molecules (pink circles), including auxin, cytokinin, proteins and RNA species, including mRNA and sRNA. These phloem-borne signaling agents complete the long-distance communication circuit that serves to integrate developmental and physiological events, occurring within shoot and root tissues, in order to optimize the plant performance under the existing growth/environmental conditions.

When soils become dry, root-derived signals are transported through the xylem to leaves in order to effect a reduction in both leaf transpiration and vegetative growth. Tight control between water uptake by the root system and the xylem transpiration stream is achieved through regulation of leaf stomatal aperture. Production of ABA within roots and its transport to the leaves could contribute to preventing excess water loss, as it is has long been known that ABA is a key regulator of stomatal conductance (Mittelheuser and Van Steveninck 1969; Schachtman and Goodger 2008).

The ABA content in roots is well correlated with both soil moisture and root relative water content (Davis and Zhang 1991; Thompson et al. 2007). Although large increases in ABA are detected in the xylem sap, when plants are exposed to drought conditions (Christmann et al. 2007), grafting studies have indicated that root-derived ABA is not necessary for drought-induced stomatal closure (Holbrook et al. 2002). Furthermore, recent studies have shown that leaf-derived synthesis of ABA contributes to water-stress-induced down-regulation of stomatal conductance (Holbrook et al. 2002; Thompson et al. 2007). Thus, further studies are required to evaluate the relative contribution of root-derived versus shoot-synthesized ABA in terms of the overall efficacy of stomatal control over the transpiration stream.

There is some indication that ethylene-based signaling may also contribute to root-to-shoot communication under abiotic stress conditions. For example, the anaerobic environment caused by soil flooding can increase the level of aminocyclopropane carboxylic acid (ACC, the immediate precursor of ethylene) in plant roots. ACC has been detected in the xylem from both flooded and drought-stressed plants (Tudela and Primo-Millo 1992; Belimov et al. 2009). This root-derived ACC is transported to the shoot where it then gives rise to increased ethylene production which can play a role in regulating shoot growth and development under these stress conditions (Voesenek et al. 2003; Pérez-Alfocea et al. 2011).

Changes in xylem sap pH have also been reported for plants exposed to drought conditions. Alkalinization of the xylem sap appears to be correlated with enhanced stomatal closure (Jia and Davies 2008; Sharp and Davies 2009). These pH changes may act synergistically with ABA and ACC to generate an effective root-to-shoot signaling system for water stress. The involvement of other known xylem-based root-to-shoot signals, such as CK, etc., remains to be established in terms of contributing to water stress signaling. In any event, advancing our understanding of the mechanisms of root-to-shoot signaling associated with water stress should lead the way for the development of crops with improved water use efficiencies.

Xylem signals associated with nutrient stress

The phenotypic plasticity that plants display in response to changes in their nutrient supply requires the operation of root-to-shoot signaling. Such signals from roots can provide shoots with an early warning of decreases in nutrient supply, while signals from shoots can ensure that the nutrient acquisition by roots is integrated to match the nutrient demand of shoots (Lough and Lucas 2006; Liu et al. 2009).

CK plays an important role in plant growth and development and its involvement as a xylem-mobile signal in regulating the nutrient starvation response, such as occurs under nitrogen and phosphorus deficiency conditions, is well established (Takei et al. 2002; Hirose et al. 2008; Ghanem et al. 2011). Nitrate deprivation leads to a reduction in the level of mobile CK in the xylem sap, whereas upon resupply of nitrate to these stress

roots, CK again increases in the xylem transpiration stream (Rahayu et al. 2005; Ruffel et al. 2011). Interestingly, trans-zeatin-type CK moves in the xylem, and isopentenyl-type CK is present in the phloem translocation stream. This suggests that these structural variations carry specific information from the root-to-shoot and shoot-to-root, respectively (Hirose et al. 2008; Werner and Schmülling 2009).

As discussed above, phosphate acquisition by the root system involves phloem-mobile signals from the shoot (**Figure 24**). In terms of the root-to-shoot component of this phosphate signaling network, it has been suggested that the level of phosphate in the xylem transpiration stream may serve as one component in this signaling pathway (Bieleski 1973, Poirier et al. 1991; Burleigh and Harrison 1999; Hamburger et al. 2002; Lai et al. 2007; Stefanovic et al. 2007; Chiou and Lin 2011; Thibaud et al. 2010). Studies on the growth of *Arabidopsis* roots being exposed to low phosphate conditions identified the tip of the primary root, including the meristem region and root cap, as the site that may sense local phosphate availability (Linkohr et al. 2002; Svistoonoff 2007). However, currently, there is no evidence for the existence of a phosphate sensor or receptor.

Both CK and SLs have also been considered to function in xylem transmission of root phosphate status (Martin et al. 2000; Franco-Zorrilla et al. 2005; Kohlen et al. 2011). Plants grown under limiting phosphate conditions have repressed levels of trans-zeatin-type CK in their xylem sap (Martin et al. 2000) and, under these conditions, expression of the CK receptor CRE1 is similarly decreased (Franco-Zorrilla et al. 2002, 2005). In many plant species, the SLs are up-regulated upon exposure to phosphate deficiency conditions. Grafting studies have indicated that SLs produced in the root can move to the shoot (Beveridge et al. 1994; Napoli 1996; Turnbull et al. 2002). In such studies, WT rootstocks grafted to mutant scions lacking the ability to produce SLs were able to restore WT branching patterns in these scions. Thus, xylem-transported SLs can contribute to the regulation of shoot architectural responses to phosphate-limiting conditions (Kohlen et al. 2011). Collectively, these findings suggest that the levels of phosphate, CK and SLs in the xylem transpiration stream play an important role in coordinating vegetative growth with phosphate nutrient availability (Rouached et al. 2011).

Xylem signaling in plant-symbiotic associations

The interaction of nitrogen-fixing bacteria (*Rhizobia*) is generally confined to legumes, whereas most flowering plants establish symbiotic associations with arbuscular mycorrhizal (AM) fungi for phosphate acquisition. In both types of plant-symbiont association, there is a significant metabolic cost to the plant host. Thus, there is a need for the plant to ensure that the cost-benefit ratio remains favorable. To this end,

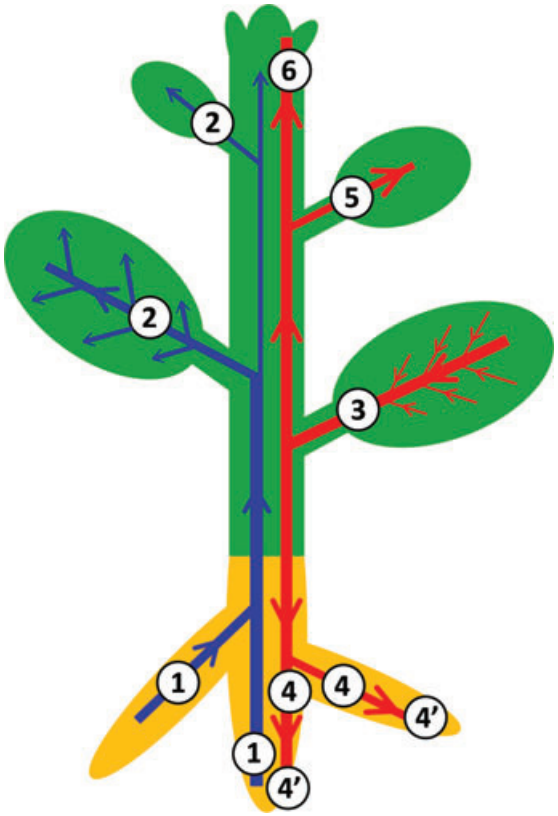

Figure 24. Long-distance signaling in response to phosphate deficiency conditions.

Phosphate (Pi) availability in the soil solution is transmitted through the xylem transpiration stream (blue lines) which passes predominantly to the mature source leaves. Pi *per se*, and/or other root-to-shoot signals (1), including cytokinin and strigolactones, are thought to be involved in this nutrient signal transduction pathway. When roots encounter low levels of available Pi, changes in these xylem-borne signaling components are decoded in the leaves (2), resulting in the activation of Pi deficiency responsive pathways. Outputs from this Pi-stress response pathway, including *miR399* 21-nucleotide silencing agents and other potential signaling components (3) are loaded into the phloem (red lines). Phloem-mobile signals move down to the root where they enter different target receiver cells to mediate an increase in Pi uptake (4) and alter root architecture (4'). The miR399 signal targets *PHOSPHATE2* (*PHO2*) transcripts to derepress Pi transporter activity. A different set of phloem-mobile signals are likely delivered to developing leaves (5) and the shoot apex (6) to regulate growth and development in order to survive under the Pi-stress condition. This long-distance signaling network operates to ensure that the root system integrates its physiological activities to optimize growth conditions within the shoot.

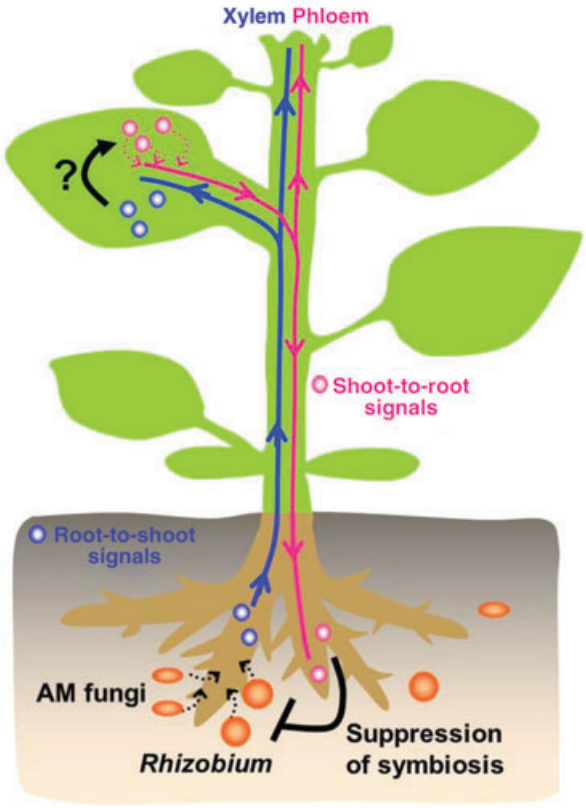

Figure 25. Autoregulation of symbiosis between plants and bacteria/fungi involves long-distance signaling through the vascular system.

During plant-symbiont associations a significant metabolic cost is incurred by the plant host. To ensure that the cost-benefit ratio remains favorable to the plant, systemic autoregulation systems evolved to control the level of root nodulation associated with nitrogen-fixing *Rhizobium* and growth on the roots of arbuscular mycorrhizal (AM) fungi. In this system, root-derived signals (blue circles) which are still unknown but may involve CLE peptides in the case of nodulation, are transported through the xylem (blue lines) to the shoot. In mature source leaves, these autoregulation of nodulation signals appear to be recognized by a LRR-RLK. Although grafting studies have established that signals (pink circles) enter and move through the phloem (pink lines) to the roots, their identities remain to be elucidated. These shoot-to-root signals are involved in down-regulating the root – *Rhizobium*/AM fungi symbiotic association.

plants have evolved a systemic feedback regulatory system termed "autoregulation" in which nodule formation and the ongoing development of the association with the AM fungus is negatively controlled by long-distance signaling (Catford et al. 2003; Staehelin et al. 2011) (**Figure 25**).

The process of autoregulation in legumes in which the number of symbiotic root nodules is controlled by long-distance communication between the root and shoot has been well studied. Two component pathways are thought to operate involving root-derived signals through the xylem and shoot-derived signals through the phloem. Following rhizobial infection, a root-derived signal is generated that is then translocated to the shoot. This xylem-borne signal is perceived in the shoot and subsequently leads to the production of a shoot-derived signal whose movement through the phloem to the roots causes a block to further nodulation. Consistent with this notion, mutant legumes have been identified that are defective in autoregulation of nodulation, and grafting experiments established that these mutants were not capable of producing the requisite shoot-derived signals.

Genetic studies suggest that CLE peptides, induced in response to rhizibial nodulation signals in roots, serve as signaling agents that travel through the xylem to the shoots. A leucine-rich repeat (LRR) CLAVATA-like receptor kinase, located in the leaves, appears to function in this signaling pathway (Searle et al. 2003). Although not yet proven, the CLE peptides imported from roots are likely perceived by the LRR autoregulation receptor kinase in the shoots (Okamoto et al. 2009; Miyazawa et al. 2010; Osipova et al. 2012). In any event, it will be of considerable interest to identify the feedback signal(s) that enters the phloem to down-regulate nodulation back in the roots (Oka-Kira et al. 2005).

In addition to CLE – LRR-RLK signals from the root, soil available nitrogen also appears to participate in this long-distance signaling system. Consistent with this notion, in legumes, high levels of soil nitrate cause strong up-regulation of root *CLE* gene expression (Okamoto et al. 2009; Reid et al. 2011). Furthermore, high nitrate or ammonia levels abolish nodulation, and autoregulation-defective mutants exhibit more or fewer nitrate-insensitive phenotypes. Thus, a combination of CLE peptides and nitrate/ammonia levels in the xylem transpiration stream could function as the root-to-shoot signals that allow legumes to integrate autoregulation of nodulation with environmental nitrogen conditions.

With respect to phosphatesignaling, studies using cucumber revealed that application of root extracts from mycorrhizal plants reduced the degree of root colonization by AM fungi (Vierheilig et al. 2003). In contrast, root extracts from non-infected plants stimulated successful AM fungal colonization. This study supports the hypothesis that an equivalent autoregulation system operates to control the plant-AM fungal association involved in plant phosphate homeostasis. Given the attributes of the cucurbits for analysis of phloem and xylem sap, this system might prove invaluable for studies aimed at identifying the agents that serve to coordinate phosphate acquisition by the roots with utilization in the shoots.

Role of xylem signals in coordination of shoot architecture

A number of studies have indicated that root-derived signals play a role in the regulation of vegetative growth (Van Norman et al. 2004; Van Norman and Sieburth 2007; Sieburth and Lee 2010). These signals contribute to water and nutrient use efficiency through the control over shoot branching and growth. The *BYPASS1, 2, 3* (*BPS1, 2, 3*) genes are required to prevent the synthesis of a novel substance, a *bps* signal that moves from root-to-shoot, where it modifies shoot growth (Van Norman et al. 2004; Van Norman and Sieburth 2007; Lee et al. 2012; Lee and Sieburth 2012).

Although the functions of the BPS proteins remain to be elucidated, studies based on *bps* mutants clearly established that the roots of these mutants cause arrested shoot growth, likely due to the over-production of an inhibitor of shoot growth. Grafting experiments established that the root system of the *bps* mutants was both necessary and sufficient to induce shoot arrest, and revealed that BPS proteins can work to generate mobile root-to-shoot signals that can inhibit shoot growth (Van Norman et al. 2004; Lee et al. 2012). It will be of great interest to unravel the underlying mechanism by which shoot growth is controlled by this signaling pathway.

Recent studies reported that *bps* mutants show normal responses to both exogenous auxin and polar auxin transport inhibitors, suggesting that the primary target of the *bps* signal is independent of auxin. Furthermore, this root-to-shoot signal appears to act in parallel with auxin to regulate patterning and growth in various tissues and at multiple developmental stages (Lee et al. 2012). Clearly, further characterization of the BPS signaling pathway could well open the door to novel approaches towards controlling shoot architecture in specialty crops.

The SLs have been referred to as rhizosphere signaling molecules (Nagahashi and Douds 2000; Akiyama et al. 2005) that also participate in the regulation of shoot architecture by suppressing lateral shoot development. Biosynthetic SL mutants exhibit a highly branching phenotype and, interestingly, phosphate starvation in these plants causes a reduction in shoot branching (Umehara et al. 2008, 2010). As previously reported, plants grown under phosphate deficiency conditions have fewer shoots and an increase in lateral roots (Umehara et al. 2010; Kohlen et al. 2011). Several plant hormones, such as auxin and ethylene, also appear to be involved in linking phosphate signaling with plant growth responses (Chiou and Lin 2011). Auxin signaling was shown to be associated with changes in root system architecture under phosphate deficiency conditions (López-Bucio et al. 2002). Recent studies suggest that an auxin receptor TRI and the auxin signaling pathway are involved in this SL-regulated root-sensing of low phosphate conditions (Mayzlish-Gati et al. 2012). It is likely that auxin and SLs function, cooperatively, to control shoot branching (Brewer et al. 2009; Domagalska and Leyser 2011). These hormones move through the xylem and phloem, respectively, to form a network of systemic signals to orchestrate plant architecture at the whole plant level.

Vascular Transport of Microelement Minerals

In the last decade, significant advances have been made in the understanding of the mechanisms that control the intracellular homeostasis of microelement minerals (Takano et al. 2008; Curie et al. 2009; Williams and Pittman 2010; Conte and Walker 2011; Waters and Sankaran 2011; Ivanov et al. 2012; Sperotto et al. 2012). However, relatively little is known about the processes governing their long-distance transport. Major questions remaining relate to the mechanisms of vascular loading/unloading, as well as the chemical speciation of these elements during their transport. Furthermore, transport is not a static process and, therefore, may differ not only with the nutrient and plant species but also with other factors, such as developmental stage, circadian cycle, and nutritional status. In this section of the review, we assess the current knowledge on microelement vascular transport focusing on these open questions.

Microelement trafficking and speciation in xylem sap

In the xylem sap, the non-proteinogenic amino acid nicotianamine (NA), histidine, and organic acids are usually associated with cationic microelements (**Figure 26**, Table 2). NA binds several transition metals with very high affinity, including, in order of stability, Fe(III), Cu(II), Ni(II), Co(II), Zn(II), Fe(II) and Mn(II) (von Wirén et al. 1999; Rellán-Álvarez et al. 2008; Curie et al. 2009). Insights into the role of NA complexation and trafficking have been provided by studies of NA-deficient mutants. Here, the *chloronerva* tomato mutant is interesting as it has high root Cu concentrations, but the concentration in xylem sap is low, indicating a failure in Cu transport into mature leaves. This finding indicates that Cu(II)-NA likely serves as a key complex in the xylem sap (Herbik et al. 1996; Pich and Scholz 1996). With regard to other cationic microelements, analysis of an *A. thaliana* nicotianamine synthase (NAS) quadruple mutant (which has low levels of NA) showed that long-distance transport of Fe through the xylem was not affected, and Fe accumulated in the leaves (Klatte et al. 2009). Other studies performed on NAS-overexpressing tobacco and *A. thaliana* plants reported elevated Ni tolerance and high Zn levels in young leaves. Finally, studies conducted on several metal hyperaccumulator species (Krämer 2010) identified Cu(II)-NA, Zn(II)-NA and Ni(II)-NA complexes in the roots, xylem sap and leaves (Schaumlöffel et al. 2003;

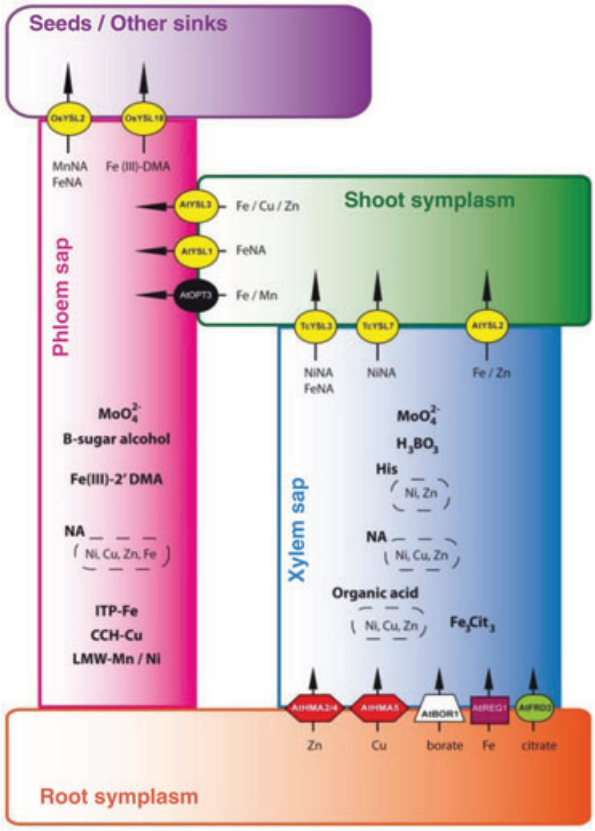

Figure 26. Schematic depicting membrane transporters involved in loading and unloading of micronutrient elements in the vascular systems.

Homologues from different plant species (At, *Arabidopsis thaliana*; Tc, *Thlaspi caerulescens*; Os, *Oryza sativa*) are given as examples. P-type ATPase (HMA), ferroportin (IREG) and and MATE. (FRD) families are involved in loading Zn and Cu, Fe, and citrate, respectively, into the xylem. Borate is loaded into the xylem by the anion efflux system, AtBOR1. The chemical species present in the xylem and phloem sap are indicated; several micronutrient species may occur in xylem sap. Histidine (His), Nicotianamine (NA), and organic acids are the most likely chelating agents of these mineral micronutrients. The complex Fe_3Cit_3 has been detected in xylem sap from tomato. Unloading of Ni, Fe and Zn from the xylem takes place via members of the Yellow Stripe-Like family of metal transporters (YSL). Phloem loading and unloading of Fe, Mn, Cu and Zn is also mediated by several members of the YSL family in rice and *Arabidopsis*. AtOPT3, a member of the oligopeptide transporter family, is involved in Fe and Mn loading into the sieve tube system. Chemical species of micronutrient minerals in the phloem sap include complexes of Ni, Cu, Zn and Fe with NA. The complexes Zn-NA and Fe (III)-2′DMA have been recently detected in phloem sap from rice. Iron Transporter Protein (ITP) and Copper Chaperone (CCH) may have a role in Fe and Cu transport within the phloem, respectively, whereas, Mn and Ni have been detected in association with low molecular (LMW) peptides and organic compounds. The molybdate anion has been detected in both xylem and phloem sap. Boron is present as borate and boric acid in xylem sap and as complexes with sugar alcohols in phloem sap.

Vacchina et al. 2003; Ouerdane et al. 2006; Mijovilovich et al. 2009, Trampczynska et al. 2010).

Histidine (His) can function to chelate Zn, Cu and Ni in the xylem sap (Krämer et al. 1996; Salt et al. 1999; Liao et al. 2000; Küpper et al. 2004). An extended X-ray absorption fine structure (EXAFS) study demonstrated that most of the Zn in petioles and stems of *Noccacea caerulescens* existed as a complex with His (Küpper et al. 2004). However, a recent study performed on the same species proposed His as a Zn ligand within cells, and NA as the Zn chelator involved in long-distance transport (Trampczynska et al. 2010). For Cu, as commented above, NA plays a key role in xylem transport. However, xylem transport of Cu in tomato and chicory is efficient even in the absence of NA, provided that His is present, thus offering support for the existence of both mechanisms for Cu complexation in xylem sap (Liao et al. 2000). Based on these findings, Irtelli et al. (2009) proposed that, under Cu deficiency conditions, NA is responsible for Cu chelation in xylem sap, whereas His and Pro serve as the major chelators in excess Cu conditions.

The involvement of His in Ni chelation in the xylem sap has been proposed based on studies in Ni-hyperaccumulator species (Krämer et al. 1996; Kerkeb and Krämer 2003; Mari et al. 2006; Krämer 2010; McNear et al. 2010). In these plants, there is an enhanced expression of the first enzyme in the His biosynthetic pathway and higher concentrations of His in xylem sap (Krämer et al. 1996; Ingle et al. 2005). On the other hand, His-overproducing transgenic *A. thaliana* lines displayed enhanced Ni tolerance, but did not exhibit increased Ni concentrations in xylem sap or leaves (Wycisk et al. 2004; Ingle et al. 2005). These studies suggest that, in non-hyperaccumulator plants, other chelating agents such as NA and organic acids, may also play important roles (Verbruggen et al. 2009; Hassan and Aarts 2011). Accordingly, studies on natural variation among *Arabidopsis* accessions indicated that a Ni(II)-malic acid complex may be involved in translocation of Ni from roots to shoots (Agrawal et al. 2012).

As mentioned above, organic acids have also been hypothesized to serve as chelators for Fe, Zn, Ni and Mn in xylem sap, based on *in silico* calculations using xylem sap composition (von Wirén et al. 1999; López-Millán et al. 2000; Rellán-Alvárez et al. 2008). For instance, *in silico* speciation studies in xylem sap of the hyperaccumulator *Alyssum serpyllifolium* found approximately 18% of Ni bound to organic acids, mainly malate and citrate (Alves et al. 2011), and in tomato, Mn was predicted

Table 2. Chemical speciation of cationic microelements present within the xylem transpiration stream and the phloem translocation stream

	Xylem sap			Phloem sap	
	Nicotianamine	Histidine	Organic acids	Nicotianamine	Peptides/proteins
Fe			Rellán-Álvarez et al. 2010	Klatte et al. 2009 Takahashi et al. 2003	ITP[a] Krüger et al. 2002
Zn	Klatte et al. 2009	Küpper et al. 2004	Salt et al. 1999 Trampczynska et al. 2010	Nishiyama et al. 2012 Takahashi et al. 2003	
Cu	Pich and Scholz 1996 Herbik et al. 1996 Liao et al. 2000		Liao et al. 2000 Irtelli et al. 2009	Pich et al. 1994	CCH Mira et al. 2001 (metallothioneins) Guo et al. 2003, 2008
Mn			White et al. 1981		LMW peptides Van Goor and Wiersma 1976
Ni	Ouerdane et al. 2006	Krämer et al. 1996 Kerkeb and Krämer 2003 Krämer 2010 McNear et al. 2010	Agrawal et al. 2012 Alves et al. 2011	Schaumlöffel et al. 2003	LMW compounds Wiersma and Van Goor 1979

[a]ITP, Iron Transport Protein; CCH, Copper Chaperone Protein; LMW, Low Molecular Weight.

as a citrate complex (White et al. 1981). However, these models did not include other possible chelating agents such as amino acids or NA. Recently, a tri-Fe(III), tri-citrate complex (Fe_3Cit_3) was identified in the xylem sap of tomato plants, using an integrated mass spectrometry approach (Rellán-Álvarez et al. 2010). Also, by means of X-ray absorption spectroscopy, organic acids have been shown to complex Zn in xylem sap of *Noccacea caerulescens* (Salt et al. 1999).

With regard to anionic microelements, the soluble molybdate anion, which is the predominant aqueous species at pH values above 4.0, has been detected in both xylem and phloem sap, and is assumed to be the major chemical species of Mo delivered by these two long-distance transport systems (Marschner 1995). The fact that the molybdate anion is not very biologically active may allow for its transport as a free anion. For boron (B), in addition to boric acid and borate, at least one other yet unidentified B-containing compound has been described in the xylem sap of squash roots (Iwai et al. 2003). This compound has a lower molecular weight than the rhamnogalacturan II-B complex, which contributes to cell wall strengthening (Takano et al. 2008).

Microelement trafficking and speciation in the phloem sap

Metal mobility in the phloem sap depends on the individual microelement, its chemical species, and in some cases on the nutritional status of the plant. Zn and Ni are considered highly mobile in the phloem translocation stream; for instance, the loading of these metals into the developing wheat grain occurs mostly via the phloem, with transfer from xylem to phloem occurring in the rachis and the peduncle (Riesen and Feller 2005). In contrast, manganese (Mn) appears to be poorly mobile in the phloem; it can be translocated out of source leaves, but the loading of Mn into the developing grain is poor in most crop species (Riesen and Feller 2005; Williams and Pittman 2010). For Cu, its mobility in the phloem sap is intermediate. For instance, in wheat, translocation from mature to younger developing leaves does not occur, and it has been proposed that when Cu enters the cell it becomes bound by chaperones and, therefore, is not immediately available for retranslocation. Later remobilization of Cu appears to be possible during leaf senescence when proteins are hydrolyzed, thereby releasing Cu (Puig and Penarrubia 2009). Fe is also considered intermediately mobile in plants, and studies have shown that it is translocated to young barley leaves mainly via phloem transport (Tsukamoto et al. 2009). Grusak (1994) also reported phloem transport of Fe from various source tissues to developing *Pisum sativum* seeds. And, as occurs with Cu, an increased remobilization of Fe occurs during leaf senescence (Waters et al. 2009; Sperotto et al. 2010).

With regard to the chemical species in which these metals are transported through the phloem (**Figure 26**), Fe, Cu and Zn are considered to move as either NA- or metal-mugineic

acid complexes (mugineic acids are synthesized from NA), especially as the neutral-to-basic pH of the phloem sap is suitable for metal-NA formation (Pich et al. 1994; von Wirén et al. 1999; Takahashi et al. 2003). Accordingly, Zn(II)-NA and Fe(III)-2′-deoxymugineic acid complexes have been detected in phloem sap from rice (Nishiyama et al. 2012). Furthermore, mutants defective in NA production display lower Mn, Zn, Fe and Cu concentrations in reproductive organs (Takahashi et al. 2003; Curie et al. 2009). However, it is still unclear whether this impediment to metal delivery into sink organs is due to alterations in phloem loading and transport, *per se*, or to changes in the intracellular concentrations of metals in source tissues where NA also plays an important role in metal chelation.

The phloem may also transport other forms of Fe; e.g., an iron transport protein (ITP) is present in the phloem sap of *Ricinus communis* (Krüger et al. 2002), but it has not yet been found in other species. A copper chaperone protein (CCH) has also been proposed to play a role in phloem transport of Cu from senescing to young leaves (Mira et al. 2001). In addition, metallothioneins (types 1, 2 and 3), proteins predominantly regulated by Cu, also appear to function in Cu accumulation and phloem transport during senescence (Guo et al. 2003, 2008). These proteins are also associated with Cu tolerance (Murphy and Taiz 1997; van Hoof et al. 2001; Jack et al. 2007). Currently, little information is available concerning the chemical forms of Ni and Mn in the phloem sap. In *R. communis*, Mn has been detected in association with low molecular weight peptides (van Goor and Wiersma 1976), whereas Ni can be complexed with negatively charged organic compounds with a molecular weight in the range of 1,000–5,000 Da (Wiersma and van Goor 1979).

The mobility of molybdenum (Mo) in the phloem varies depending on its concentration and on plant age. Interestingly, in wheat, Mo has been associated with the existence of "Mo-binding sites" in the phloem that, until saturated, appear to prevent its long-distance translocation (Yu et al. 2002). B mobility in the phloem is highly dependent on the plant species. In plants that transport sugar alcohols, B appears to be complexed with diols and polyols (Brown and Hu 1996; Hu et al. 1997; Takano et al. 2008). Complexes of sorbitol-B-sorbitol, fructose-B-fructose, sorbitol-B-fructose and mannitol-B-mannitol have been identified in peach and celery phloem sap (Hu et al. 1997). Enhancement of sorbitol production results in an increase of B translocation from mature leaves to sink tissues as well as tolerance to B deficiency. In plant species that do not produce significant amounts of sugar alcohols, B is thought to be phloem immobile, or only slightly mobile, and its distribution in shoots seems primarily to follow the xylem transpiration stream (Oertli 1993; Bolaños et al. 2004; Lehto et al. 2004; Takano et al. 2008).

Vascular loading and unloading of cationic micronutrients

Yellow Stripe-Like (YSL) proteins play important roles in the short- and long-distance transport of microelements and their delivery to sink tissues (Curie et al. 2009). Members of the YSL family, AtYSL1, AtYSL2, AtYSL3, OsYSL2 and OsYSL18, are expressed in vascular tissues (Table 3) and may have a role in the lateral movement of Fe within the veins and in phloem transport (DiDonato et al. 2004; Koike et al. 2004; Le Jean et al. 2005; Schaaf et al. 2005; Aoyama et al. 2009). The rice OsYSL2 can transport Fe(II)-NA and Mn(II)-NA to an equal extent (Koike et al. 2004). OsYSL18 transports Fe(III)-deoxymugineic acid (Aoyama et al. 2009), whereas there are contradictory reports concerning the ability of AtYSL2 to transport Fe(II)-NA and Cu(II)-NA (DiDonato et al. 2004; Schaaf et al. 2005). AtYSL1 seems to play a role in Fe(II)-NA translocation to seeds (Le Jean et al. 2005). A study on the *Arabidopsis* double mutant *ysl1ysl3* reported reduced accumulation of Fe, Cu and Zn in seeds, consistent with involvement of the YSL1 and YSL3 transporters in remobilization from leaves (Waters et al. 2006). There is also evidence for a role of YSLs in the Zn and Ni hyperaccumulation of *Thlaspi caerulescens*, especially for TcYSL3 and TcYSL7, which are highly expressed around vascular tissues particularly in shoots when compared with their *A. thaliana* orthologs (Gendre et al. 2007). TcYSL3 is an Fe(II)-NA and Ni(II)-NA influx transporter that is suggested to facilitate the movement of these metal-NA complexes from the xylem into leaf cells.

A number of other transporters involved in vascular loading and unloading of microelements have also been identified (**Figure 26**, Table 3). The *Arabidopsis* OPT3 transporter (OligoPeptide Transporter) appears to be essential for embryo development (Stacey et al. 2008). This protein transports Mn, and its expression in the vascular tissue suggests a role in Mn long-distance transport. Although yeast studies have suggested that it can also transport Cu, OPT3 does not appear to play a role in Zn or Cu loading, as seeds of *opt3-2* plants actually accumulate increased levels of these two metals (Stacey et al. 2008). The *opt3-2* mutant also has reduced Fe concentrations in its seeds as well as impaired seedling growth under Fe-deficient conditions, thus suggesting a role in Fe loading into the seed (and perhaps even phloem-mediated redistribution).

Another Fe efflux transporter, IREG1/FPN1 (Iron Regulated1/Ferroportin1), is considered to function in Fe loading into the xylem within the roots (Morrissey et al. 2009). Loss of *FPN1* function results in chlorosis, and FPN1-GUS plants show staining at the plasma membrane of the root vascular system. However, yeast complementation studies using FPN1 have failed, and information on the chemical form of Fe

Table 3. Transporters involved in vascular loading and unloading of cationic microelements

Family	Gene	Microelement	Function	Reference
YSL	*AtYSL1*	Fe-NA	Seed loading, phloem loading	Le Jean et al. 2005
	AtYSL2	Fe, Zn	Xylem loading/unloading	DiDonato et al. 2004 Schaaf et al. 2005
	AtYSL3	Fe, Cu, Zn	Phloem loading	Waters et al. 2006
	OsYSL2	Fe-NA Mn-NA	Phloem loading/unloading to grains	Koike et al. 2004
	OsYSL18	Fe(III)-DMA	Phloem loading/unloading to reproductive organs	Aoyama et al. 2009
	TcYSL3	Fe-NA, Ni-NA	Xylem unloading	Gendre et al. 2007
	TcTSL7	Ni-NA	Xylem unloading	Gendre et al. 2007
OPT	*AtOPT3*	Mn Fe	Seed loading	Stacey et al. 2008
Ferroportin	*IREG1*/*FPN1*	Fe	Xylem loading	Morrissey et al. 2009
MATE	*FRD3*	Citrate	Xylem loading	Durrett et al. 2007 Yokosho et al. 2009 Yokosho et al. 2010
P-type ATPases	*AtHMA5*	Cu	Xylem loading	André-Colás et al. 2006 Kobayashi et al. 2008
	AtHMA2/*4*	Zn	Xylem loading	Hussain et al. 2004 Mills et al. 2005 Wong and Cobbett 2009 Verret et al. 2004, 2005
	AhHMA4	Zn	Xylem loading	Hanikenne et al. 2008

transported by FPN1 has yet to be established (Morrissey et al. 2009).

The *Arabidopsis* P-type ATPases, AtHMA5 and AtHMA2/4, have been implicated in Cu and Zn efflux, respectively, into the xylem at the root level, for long-distance transport to the shoots (Hussain et al. 2004; Mills et al. 2005; Andrés-Colas et al. 2006). Consistent with this model, both *hma5* and *hma2hma4* loss-of-function mutants accumulate increased levels of the corresponding metal within the root, and show lower levels in their shoots (Hanikenne et al. 2008; Wong and Cobbett 2009). HMA5 is predominantly expressed in the root and is specifically induced by excess Cu. Mutants of HMA5 overaccumulate Cu in the root, suggesting a compromised efflux system. Further evidence in support of the role of HMA5 in xylem transport of Cu from the roots to the shoots comes from a study of natural variation in Cu tolerance among *Arabidopsis* accessions, which identified HMA5 as a major QTL associated with Cu translocation capacity and sensitivity (Kobayashi et al. 2008). HMA2 and 4 are present in the plasma membrane of root and shoot vascular tissues (Mills et al. 2003; Hussain et al. 2004; Verret et al. 2004; Mills et al. 2005; Verret et al. 2005; Williams and Mills 2005; Sinclair et al. 2007; Blindauer and Schmid 2010). In addition, functional analysis of HMA4 in *A. halleri* and *A. thaliana* showed that silencing of *AhHMA4*, by RNA interference, completely suppressed Zn hyperaccumulation. These studies provided a clear demonstration that HMA4 plays a key role in xylem loading and, consequently, in root-to-shoot transport of Zn (Hanikenne et al. 2008).

Organic acids may also have a role in xylem Fe loading. Citrate has been described as an Fe(III) chelator in the xylem sap (Rellán-Álvarez et al. 2010) and FRD3 (Ferric Reductase Defective), a transporter of the MATE family, is localized to the plasma membrane of the pericycle and vascular cylinder. FRD3 proteins facilitate citrate efflux into the xylem of the root vasculature and have been described in *Arabidopsis* (Durrett et al. 2007), rice (Yokosho et al. 2009) and rye (Yokosho et al. 2010). Mutant *frd3* plants are chlorotic, show reduced citrate and Fe concentrations in the xylem and the shoot, accumulate Fe in the root, and exhibit constitutive expression of the Fe uptake components, thus suggesting that FRD3 is necessary for efficient Fe transport to the shoot through the transpiration stream. Also, independent Fe-citrate and Fe-NA xylem loading systems may complement each other, as in the *frd3* mutant, the nicotianamine synthase NAS4 gene is induced, and the double mutant *nas4x-2*/*frd3* shows impaired growth and low Fe levels in the shoot (Schuler et al. 2010). FRD3 is constitutively expressed in the hyperaccumulators *A. halleri* and *N. caerulescens* compared to *A. thaliana* and *N. arvensis*, and may also play a role in Zn transport (Talke et al. 2006; van de Mortel et al. 2006). However, this overexpression may be related to an altered Fe homeostasis leading to high Zn concentrations in the hyperaccumulators (Roschzttardtz et al. 2011).

Vascular loading and unloading of anionic micronutrients

Xylem loading of B is mediated by BOR1 (Takano et al. 2001; Miwa and Fujiwara 2010). BOR1 is an anion efflux system that is strongly expressed in the root pericycle cells surrounding the xylem vessels. The *bor1-1* mutant is defective in xylem loading of B (Takano et al. 2002). The specific chemical form of the substrate for BOR1 remains unknown, but electrophysiological analyses in the human homolog, NaBC1, suggest borate anion as the likely candidate (Park et al. 2004). There are six BOR1 paralogs in the *A. thaliana* genome which may also have roles in xylem-phloem loading and unloading (Miwa and Fujiwara 2010). A second B transporter, NIP6,1, is a channel protein required for proper distribution of boric acid, particularly to young developing shoot tissues (Tanaka et al. 2008). This transporter is predominantly expressed in nodal regions of shoots, especially the phloem region of vascular tissues, where it is likely involved in xylem-phloem transfer of boric acid.

The mechanisms for Mo loading and unloading in the vascular tissues remain to be elucidated. To date, only one Mo transporter from *Arabidopsis*, MOT1, has been identified in plants. MOT1 is a high-affinity molybdate transporter localized to the plasma membrane or mitochondrial membranes, and plays an important role in efficient Mo uptake from soils and accumulation within the plant (Tomatsu et al. 2007; Baxter et al. 2008). MOT1 belongs to the family of sulphate transporters, SULTR (Hawkesford 2003), which has 14 members in *A. thaliana*. It is tempting to speculate that some of these transporters may be involved in vascular tissue loading or unloading.

Systemic Signaling: Pathogen Resistance

Like all living organisms, plants have to constantly resist pathogenic microbes. The absence of a circulatory vascular system and their sessile nature can pose particular problems. Plants have therefore evolved unique defense mechanisms to ensure survival. The multiple modes of plant defense include both passive and active mechanisms that provide defense against a wide variety of pathogens. Active defense includes the production of antimicrobial compounds, cell wall reinforcement via the synthesis of lignin and callose, and the specific induction of elaborate defense signaling pathways. These include species level (non-host) resistance, race-specific resistance expressed both locally and systemically, and basal resistance.

Race-specific resistance is induced when strain-specific avirulent (Avr) proteins from the pathogen associate directly/indirectly with cognate plant resistance (R) proteins (reviewed in Jones and Dangl 2006; Caplan et al. 2008). Induction of R-mediated signaling is often accompanied by the onset of a hypersensitive response (HR), a form of PCD resulting in necrotic lesions, at the site of pathogen entry (Dangl et al. 1996). HR is one of the first visible manifestations of pathogen-induced host defenses, and is thought to help confine the pathogen to the dead cells. R-mediated signaling is also often accompanied by the induction of a robust form of resistance against secondary pathogens in the systemic parts of the plants, termed systemic acquired resistance (SAR) (Durrant and Dong 2004; Vlot et al. 2008; Spoel and Dong 2012).

Identified as a form of plant immunity nearly 100 years ago, SAR is a highly desirable form of resistance that protects against a broad-spectrum of pathogens. SAR involves the generation of a mobile signal at the site of primary infection, which moves to and arms distal portions of a plant against subsequent secondary infections (**Figure 27**). The identification of this signal could greatly facilitate the use of SAR in protecting agriculturally important plants against a wide range of pathogens. Because of its unique mechanistic properties and its exciting potential applications in developing sustainable crop protection strategies, SAR has been one of the most intensely researched areas of plant biology. The last decade has witnessed considerable progress, and a number of signals contributing to SAR have been isolated and characterized. Despite concerted efforts to harness this mode of plant immunity, the plant defense field lacks a consensus regarding the identity of the SAR signal, whether this signal constitutes multiple molecular components, and how these component(s) might coordinate the systemic induction of broad-spectrum resistance.

Among the signals contributing to SAR are salicylic acid (SA) and several components that feed into the SA pathway, including the methylated derivative of SA (MeSA; Park et al. 2007), the diterpenoid dehdryoabietinal (DA; Chaturvedi et al. 2012), the nine carbon (C9) dicarboxylic acid azelaic acid (AA; Jung et al. 2009), auxin (Truman et al. 2010), the phosphorylated sugar glycerol-3-phosphate (G3P; Chanda et al. 2011; Mandal et al. 2011), and two lipid transfer proteins (LTPs), Defective in Induced Resistance (DIR1; Maldonado et al. 2002) and AA insensitive (AZI1; Jung et al. 2009). Jasmonic acid (JA) has also been suggested to participate in SAR (Truman et al. 2007), but its precise role remains contentious (Attaran et al. 2009). The diverse chemical natures of the SAR-inducing molecules have led to the growing belief that SAR might involve the interplay of multiple diverse and independent signals. In this final section of the review, we will evaluate the role of SA and the recently identified mobile inducers of SAR.

SA and SAR

SA is a central and critical component of SAR. The biosynthesis of SA occurs via the shikimic acid pathway, which bifurcates into two branches after the biosynthesis of chorismic acid. In one branch, chorismic acid is converted to SA via

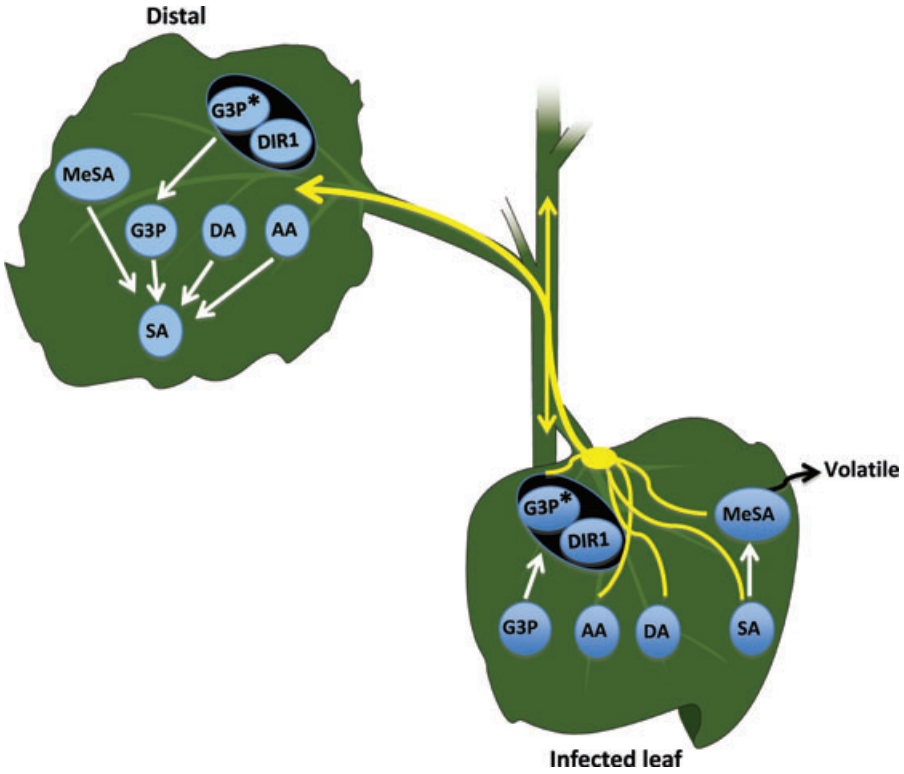

Figure 27. A simplified model summarizing the known mobile inducers of systemic acquired resistance (SAR).

Pathogen infection induces an increase in the levels of glycerol-3-phosphate (G3P), azelaic acid (AA), dehdryoabietinal (DA), salicylic acid (SA) and methyl SA (MeSA). Some of the SA is converted to MeSA, whereas G3P is converted to an unknown derivative (indicated by asterisk). Owing to its volatile nature, a significant proportion of the MeSA is thought to escape by emissions. Although both SA and MeSA are phloem mobile, SA likely functions downstream of mobile signal generation. This is based on grafting experiments that indicate SA is not the SAR signal, yet basal SA is essential for G3P-, DA-, and AA-mediated SAR. DA induces SA accumulation in infected and distal tissues, whereas AA primes for SA biosynthesis in response to secondary pathogen stimulus. Neither G3P nor AA induces SA accumulation. G3P-, AA-, and DA-mediated SAR require the endoplasmic reticulum-localized lipid transfer-like protein, DIR1. Systemic movement of G3P and DIR1 is mutually inter-dependent. Upon transport, complex(es) comprising DIR1 and the G3P-derivative induce *de novo* G3P biosynthesis in the distal tissues.

phenylalanine and cinnamic acid intermediates, and in the other branch chorismic acid is converted to SA via isochorismic acid. Two well characterized enzymes in these branches include PHENYLALANINE AMMONIA LYASE (PAL), which converts phenylalanine to cinnamic acid, and ISOCHORISMATE SYNTHASE (ICS), which catalyzes the conversion of chorismic acid to isochorismic acid (Wildermuth et al. 2001; Strawn et al. 2007).

Transcriptional profiling has shown that expression of hundreds of genes is altered during the development of SAR (Schenk et al. 2000; Wang et al. 2006; Truman et al. 2007; Chanda et al. 2011). This is likely to have wide-ranging effects, including strengthening of the cell wall and production of reactive oxygen species and SA. A hallmark of plants that have manifested SAR is the induction of pathogenesis-related (PR) proteins (Carr et al. 1987; Loon et al. 1987; Ward et al. 1991). These observations and the fact that exogenous SA induces PR expression led to the suggestion that SA was involved in SAR signaling.

Exogenous application of SA or its synthetic functional analogues such as BTH (1,2,3-benzothiadiazole-7-carbothioic acid, S-methyl ester) also induce generalized defense against a variety of pathogens. Evidence supporting a role for SA in plant defense came from analysis of transgenic plants expressing the bacterial gene encoding salicylate hydroxylase, an enzyme that catalyzes conversion of SA to catechol. These transgenic plants were unable to accumulate free SA, showed compromised defense, and were unable to induce SAR (Gaffney et al. 1993; Friedrich et al. 1995; Lawton et al. 1995). The fact that pathogen inoculation induces SA accumulation in both local and distal uninoculated tissues led to the hypothesis that SA might well be the phloem-mobile signal (Vernooij et al. 1994;

Shulaev et al. 1995). However, the timeframe of SAR signal movement precedes that of SA accumulation in the distal tissues. Moreover, SA-deficient rootstocks of plants expressing SA hydroxylase or plants suppressed in PAL expression are capable of activating SAR in the leaves of WT scions. These data argued against a role for SA as the phloem-mobile SAR signal (Vernooij et al. 1994; Pallas et al. 1996). Regardless, SA is required for the proper induction of SAR, and mutations in either the ICS or PAL pathway are sufficient to compromise SAR (Vernooij et al. 1994; Pallas et al. 1996; Wildermuth et al. 2001).

Radiolabel feeding experiments suggest that over 50% of SA in distal leaves is transported from the inoculated leaves with the remainder being synthesized *de novo* (Shulaev et al. 1995; Molders et al. 1996). Whether the induced synthesis of SA in the distal tissues is required for SAR remains unclear. A marginal difference (~10 ng/g FW) in SA levels between SAR-competent WT versus SAR-compromised SA-deficient scions grafted onto SA-deficient rootstocks suggests that an increase in SA accumulation may not be a prerequisite for the normal induction of SAR (Vernooij et al. 1994; Chanda et al. 2011).

In general, most of the endogenous SA is metabolized to a glucose conjugate, SA 2-*O*-β-D-glucose (SAG) or SA glucose ester (SGE), and this reaction is catalyzed by SA glucosyltransferases (Enyedi et al. 1992; Edwards 1994; Lee and Raskin 1998; Lee and Raskin 1999; Dean and Delaney 2008; Song et al. 2008). Other derivates of SA include its methylated ester and methyl SA. (MeSA), and hydroxylated form gentisic acid (GA), both of which are also present as glucose conjugates. Exogenous GA induces a specific set of PR proteins in tomato that are not induced by SA, suggesting that SA and GA differ in their mode of action (Bellés et al. 1999).

Unlike SA, MeSA is biologically inactive and only functions when converted back to SA. MeSA is a well-characterized volatile organic compound that can function as an airborne defense signal and can mediate plant-plant communication (Shulaev et al. 1997; Koo et al. 2007). Conversion of SA to MeSA is mediated by SA methyltransferases (SAMT), also designated BA (benzoic acid)-/SA-MT because it can utilize either SA or BA as substrates (Chen et al. 2003; Effmert et al. 2005; Koo et al. 2007). Overexpression of BSMT leads to the depletion of endogenous SA and SAG, as most of the available SA is converted to MeSA (Koo et al. 2007). This in turn is associated with increased susceptibility to bacterial and fungal pathogens, suggesting that levels of free SA, but not MeSA, are critical for plant immunity. Likewise, overexpression of the *Arabidopsis* SA glucosyltransferase (AtSGT1) also results in the depletion of SA and an increase in MeSA levels, which again correlates with increased susceptibility to bacterial pathogens (Song et al. 2008).

MeSA accumulates in the phloem following induction of SAR, and this requires SAMT activity. Upon translocation to the distal tissues, MeSA is converted back to SA via MeSA esterase (**Figure 27**). Most of the MeSA accumulating in response to pathogen inoculation was shown to escape by volatile emissions (Attaran et al. 2009). Furthermore, *Arabidopsis* BSMT mutant plants do not accumulate MeSA, but remain SAR competent. This discrepancy was attributed to the dependency of MeSA-derived signaling on light (Liu et al. 2011), which is well-known to play an important role in plant defense (Karpinski et al. 2003; Roberts and Park 2006). Notably, the phloem translocation time of the SAR signal to distal tissues precedes the time of MeSA requirement; i.e., 48 h and 72 h post primary infection, respectively (Park et al. 2009; Chanda et al. 2011; Chaturvedi et al. 2012). This suggests that MeSA is unlikely to be the primary mobile signal, and possibly might act as a downstream contributor to SAR.

Recent studies also suggest that defective SAR in *dir1* plants is associated with increased expression of BSMT1, which correlates with increased accumulation of MeSA and a reduction in SA and SAG levels (Liu et al. 2011). However, this is in contrast with two other independent studies that showed normal SA levels in pathogen inoculated *dir1* plants (Maldonado et al. 2002; Chaturvedi et al. 2012). Some possibilities that might account for these discrepancies are disparate regulation of BSMT1 expression and the associated changes in MeSA and SA levels in different ecotypic backgrounds, and/or plant growth conditions, such as light, humidity, temperature, and wind. For example, light intensities could affect SA levels/defense responses since photoreceptors are well known to regulate both SA- and R-mediated signaling (Genoud et al. 2002; Jeong et al. 2010).

Components that affect SAR by regulating SA levels

Many proteins known to mediate SA-derived signaling have been identified as contributors to SAR. These include proteins involved in SA biosynthesis (including ICS and PAL), transport, and/or SA-dependent R-mediated signaling (ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), EDS5, PHYTOALEXIN DEFICIENT 4 (PAD4), and SENESCENCE-ASSOCIATED gene 101 (SAG101)). The *Arabidopsis* EDS5 (also called SA INDUCTION-DEFICIENT 1) encodes a plastid-localized protein that shows homology to the bacterial multidrug and toxin extrusion transporter (MATE) proteins. EDS5 is required for the accumulation of SA after pathogen inoculation (Nawrath et al. 2002; Ishihara et al. 2008) and, consequently, a mutation in EDS5 causes enhanced susceptibility against oomycete, bacterial, and viral pathogens (Rogers and Ausubel 1997; Nawrath et al. 2002; Chandra-Shekara et al. 2004). Mutations in ICS1 and EDS5 lead to similar phenotypes (Venugopal et al. 2009), suggesting that EDS5 might be involved in

the transport of SA and/or its precursors across the plastid membrane.

Currently, EDS5 is thought to act downstream of three other signaling components, EDS1 and PAD4, and NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1), which are required for basal R protein-mediated signaling and the SAR response (Glazebrook and Ausubel 1994; Century et al. 1995; Glazebrook et al. 1996; Parker et al. 1996; Century et al. 1997; Aarts et al. 1998; Zhou et al. 1998; Shapiro and Zhang 2001; Liu et al. 2002; Coppinger et al. 2004; Hu et al. 2005; Truman et al. 2007). Some of the *Arabidopsis* ecotypes express two functionally redundant isoforms of EDS1 which interact with each other as well as with the structurally similar PAD4 and SAG101 proteins (Feys et al. 2001; He and Gan 2002; Feys et al. 2005; García et al. 2010; Zhu et al. 2011). EDS1, PAD4, and SAG101 proteins also exist as a ternary complex (Zhu et al. 2011).

EDS1 interacts with several R proteins, suggesting that EDS1 and, by extension, PAD4 and SAG101, likely act at the R protein level (Bhattacharjee et al. 2011; Heidrich et al. 2011; Zhu et al. 2011). Notably, mutations in EDS1, PAD4, and SAG101 lead to overlapping as well as independent phenotypes, suggesting that these proteins might function as complex(s) as well as individual proteins. Mutations in EDS1 or PAD4 attenuate the expression of FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1), which is required for SA accumulation in the distal tissues and, thereby, SAR (Mishina and Zeier 2006). The pathogen-induced SA levels are also regulated by AGD2-LIKE DEFENSE 1 (ALD1), which is induced in distal tissues after avirulent inoculation in a PAD4-dependent manner (Song et al. 2004a). The ALD1 encoded protein shows aminotransferase activity *in vitro*, suggesting that an amino acid-derived signal might participate in the regulation of SA levels and, thereby, SAR (Song et al. 2004b).

SA signaling components that affect SAR

The NON-EXPRESSOR OF PR1 (NPR1), an ankyrin repeat containing protein also called NON-INDUCIBLE IMMUNITY 1 (NIM1; Delaney et al. 1995; Ryals et al. 1997) or SA INSENSITIVE 1) (SAI1; Shah et al. 1997), is considered a central regulator of SA-derived signaling. A mutation in *Arabidopsis* NPR1 abolishes SAR, suggesting that it is a positive regulator of SAR (Cao et al. 1994; Cao et al. 1997). Besides SA signaling and SAR, NPR1 also functions in induced systemic resistance (ISR) and, possibly, in regulating cross-talk between the SA and jasmonic acid (JA) pathways (van Wees et al. 2000; Kunkel and Brooks 2002; Lavicoli et al. 2003; Spoel and Dong 2008). SA and NPR1 negatively affect the symbiotic interaction between Medicago and Rhizobium (Peleg-Grossman et al. 2009), suggesting that SA and NPR1 are essential components of multiple signaling pathway(s).

In the absence of SA, NPR1 exists as an oligomer via intermolecular disulfide bonding, and remains in the cytoplasm (Mou et al. 2003). Reducing conditions triggered upon activation of defense responses and the accumulation of SA result in the dissociation of the NPR1 oligomer into monomers, which are transported into the nucleus (Mou et al. 2003; Tada et al. 2008). Within the nucleus, these NPR1 monomers interact with members of the TGACG motif binding transcription factors belonging to the basic leucine zipper (bZIP) protein family (Zhang et al. 1999; Després et al. 2000; Niggeweg et al. 2000; Zhou et al. 2000; Chern et al. 2001; Fan and Dong 2002; Kim and Delaney 2002). SA also induces the reduction of the disulfide bridges in TGA proteins, thereby allowing the proteins to interact with NPR1 with subsequent activation of gene expression (Després et al. 2003).

Genetic evidence supporting a role for TGA factors in SAR was provided by the analysis of the *tga2 tga5 tga6* triple mutant, which was unable to induce PR gene expression in response to SA and was defective in the onset of SAR (Zhang et al. 2003). Recent studies have also shown that, like NPR1, TGA1 also undergoes S-nitrosylation, which promotes the nuclear translocation of NPR1 and increases the DNA binding activity of TGA1 (Tada et al. 2008; Lindermayr et al. 2010).

The monomerization of NPR1 also appears to be important for the activation of the NPR1 regulated members of the WRKY transcription factor family (Mou et al. 2003; Wang et al. 2006). In addition, NPR1 controls the expression of the protein secretory pathway genes in a TGA2-, TGA5- and TGA6-independent manner (Wang et al. 2006). The nuclear NPR1 is phosphorylated and recycled in a proteasome-dependent manner (Spoel et al. 2009). This turnover is required for the establishment of SAR. The *Arabidopsis* genome contains five paralogs of NPR1 (Liu et al. 2005). Like NPR1, NPR3 and NPR4 also interact with TGA proteins (Zhang et al. 2006). The *npr3 npr4* mutant plants accumulate higher levels of NPR1 and, consequently, are unable to induce SAR.

In a recent study, NPR3 and NPR4 were shown to bind SA and to function as adaptors of the Cullin 3 ubiquitin E3 ligase to mediate NPR1 degradation in an SA-dependent manner (Fu et al. 2012). NPR3 and NPR4 are neither the first nor the only known SA binding proteins. However, much like most of the plant hormone receptors, NPR3 and NPR4 are the only known proteins which regulate the proteasome-dependent recycling of a master regulator of the SA-signaling pathway. For this reason, these proteins have been suggested to serve as the long-sought-after SA receptors. In yet another study, NPR1 was also proposed to function as a SA receptor (Wu et al. 2012). Like NPR3/NPR4, NPR1 bound SA and the kinetics of this binding were similar to those of other receptor-hormone interactions. Thus, SA might well bind to multiple NPRs and differentially modulate their function(s).

Other proteins that bind SA include the MeSA esterase SABP2 (Kumar and Klessig 2003). Binding of SA to SABP2 inhibits its esterase activity, resulting in the accumulation of MeSA. In addition, SA binds catalase (Chen et al. 1993) and carbonic anhydrase (Slaymaker et al. 2002), and inhibits the activities of the heme-iron-containing enzymes catalase, ascorbate peroxidase, and aconitase (Durner and Klessig 1995). The ability of SA to chelate iron has been suggested as one of the mechanisms for SA-mediated inhibition of these enzymes (Rueffer et al. 1995). Tobacco plants silenced for carbonic anhydrase or aconitase show increased pathogen susceptibility, suggesting that these proteins are required for plant defense (Slaymaker et al. 2002; Moeder et al. 2007).

Whereas NPR1 is an essential regulator of SA-derived signaling, many protein-induced pathways are known to activate SA-signaling in an NPR1-independent manner (Kachroo et al. 2000; Takahashi et al. 2002; Raridan and Delaney 2002; Van der Biezen et al. 2002). Furthermore, a number of mutants have been isolated that induce defense SA signaling in an NPR1-independent manner (Kachroo and Kachroo 2006). A screen for *npr1* suppressors resulted in the identification of SNI1 (SUPPRESSOR OF npr1, INDUCIBLE), a mutation which restores SAR in *npr1* plants by de-repressing NPR1-dependent SA responsive genes (Li et al. 1999; Mosher et al. 2006). SNI1 has been suggested to regulate recombination rates through chromatin remodeling (Durrant et al. 2007). A subsequent screen for *sni1* suppressors identified BRCA2 (BREAST CANCER) and RAD51D, which when mutated abolish the sni1-induced de-repression of NPR1-dependent gene expression (Durrant et al. 2007; Wang et al. 2010). SNI1 is therefore thought to act as a negative regulator that prevents recombination in the uninduced state. A role for SNI1, BRCA2 and RAD51D in recombination and defense suggests a possible link between these processes. Collectively, such findings support a key role for chromatin modification in the activation of plant defense and SAR (March-Díaz et al. 2008; Walley et al. 2008; Dhawan et al. 2009; Ma et al. 2011).

Mobile inducers of SAR

Recent advances in the SAR field have led to the identification of four mobile inducers of SAR, including MeSA, AA, DA and G3P. All of these inducers accumulate in the inoculated leaves after pathogen inoculation and translocate systemically (**Figure 27**). The role of MeSA, a methylated derivative of SA, was discussed above. The dicarboxylic acid AA and the diterpenoid DA induce SAR in an ICS1-, NPR1-, DIR1-, and FMO1-dependent manner (Jung et al. 2009; Chaturvedi et al. 2012). Their common requirements for these components suggest that AA- and DA-mediated SAR may represent different branches of a common signaling pathway. Indeed, exogenous application of low concentrations of DA and AA, that do not activate SAR, do so when applied together. However, AA and DA differ in their mechanism of SAR activation: DA increases SA levels in local and distal tissues, whereas AA primes for pathogen-induced biosynthesis of SA in the distal tissues. DA application also induces local accumulation of MeSA. Unlike DA, AA does not induce SA biosynthesis when applied by itself. This is intriguing, considering their common requirements for downstream factors. At present, the biosynthetic pathways for AA and DA and the biochemical basis of AA- and DA-induced SAR remain unclear. Furthermore, firm establishment of AA or DA as mobile SAR inducers awaits the demonstration that plants unable to synthesize these compounds are defective in SAR.

G3P is a phosphorylated three-carbon sugar that serves as an obligatory component of glycolysis and glycerolipid biosynthesis. In the plant, G3P levels are regulated by enzymes directly/indirectly involved in G3P biosynthesis, as well as those involved in G3P catabolism. Recent results have demonstrated a role for G3P in R-mediated defense leading to SAR and defense against the hemibiotrophic fungus *Colletotrichum higginsianum* (Chanda et al. 2008). *Arabidopsis* plants containing the RPS2 gene rapidly accumulate G3P when infected with an avirulent (Avr) strain of the bacterial pathogen *Pseudomonas syringae* (avrRpt2); G3P levels peak within 6 h post-inoculation (Chanda et al. 2011). Strikingly, accumulation of G3P in the infected and systemic tissues precedes the accumulation of other metabolites known to be essential for SAR (SA, JA).

Mutants defective in G3P synthesis are compromised in SAR, and this defect can be restored by the exogenous application of G3P (Chanda et al. 2011). Exogenous G3P also induces SAR in the absence of primary pathogen, albeit only in the presence of the LTP-like protein DIR1, which is a well-known positive regulator of SAR (Maldonado et al. 2002; Champigny et al. 2011; Chanda et al. 2011; Liu et al. 2011; Chaturvedi et al. 2012). DIR1 is also required for AA- and DA-mediated SAR, suggesting that DIR1 might be a common node for several SAR signals. Interestingly, G3P and DIR1 are interdependent on each other for their translocation to the distal tissues. However, G3P does not interact directly with DIR1. Moreover, ^{14}C-G3P-feeding experiments have shown that G3P is translocated as a modified derivative during SAR. These results suggest that DIR1 likely associates with a G3P-derivative and, upon translocation to the distal tissues this complex, then induces the *de novo* synthesis of G3P and consequently SAR (**Figure 27**).

This defense-related function of G3P is conserved because exogenous G3P can also induce SAR in soybean (Chanda et al. 2011). Exogenous application of G3P on local leaves induces transcriptional reprogramming in the distal tissues, which among other changes leads to the induction of the gene encoding a SABP2-like protein and repression of BSMT1. Thus, it is possible that G3P-mediated signaling functions to prime

the system for SA biosynthesis in the presence of an invading pathogen. However, exogenous G3P alone is not associated with increased SA biosynthesis, in either local or distal leaves. In this regard, it is interesting that similar to G3P, AA does not induce the expression of the genes normally associated with SA signaling, or those induced in response to exogenous SA. Induced SA accumulation diverts carbon, nitrogen and energy away from the plant's primary metabolic pathways, which negatively impacts growth and development (Heil and Baldwin 2002; Heidel et al. 2004). Thus, chemicals like AA and G3P, which induce SAR without increasing SA levels, could be tremendously beneficial in improving crop resistance without affecting plant growth, development and ultimately yield.

Fatty acids, lipids, cuticle and plant defense

The primary role of G3P in plant metabolism is that of an obligatory precursor for glycerolipid biosynthesis. G3P enters lipid biosynthesis upon acylation with the fatty acid (FA) oleic acid (18:1) to form lyso-phosphatidic acid (lyso-PA), via the activity of the soluble plastidial G3P acyltransferase (GPAT). Genetically-based reductions in 18:1 levels induce constitutive defense signaling via the SA pathway (Kachroo et al. 2003, 2004, 2005; Venugopal et al. 2009). Consequently, low 18:1-containing plants exhibit enhanced resistance to bacterial and oomycete pathogens (Shah et al. 2001; Kachroo et al. 2001). Low 18:1 levels also specifically induce the expression of several R genes, which in turn induces defense signaling. SA and EDS1 regulate this low 18:1-dependent induction of defense responses in a redundant manner (Chandra-Shekara et al. 2007; Venugopal et al. 2009; Xia et al. 2009). Interestingly, it has also been shown that 18:1 levels regulate the NOA1 (NITRIC OXIDE ASSOCIATED) protein and thereby nitric oxide levels. Thus, the increased NO in low 18:1-containing plants is responsible for their altered defense related phenotypes (Mandal et al. 2012).

A number of cuticle-defective mutants are compromised in SAR (Xia et al. 2009, 2010). Whereas *acp4* plants can generate the signal required for inducing SAR, they are unable to respond to it. This loss of ability to "perceive" the SAR signal appears to be related to the defective cuticle of *acp4* plants, because mechanical abrasion of the cuticle disrupts SAR in WT plants. This SAR-disruptive effect of cuticle abrasion is highly specific because it hinders SAR only during the time-frame of mobile signal generation and translocation to distal tissues; it does not alter local defenses. These observations suggest that cuticle-derived component(s) likely participate in processing/perception of the SAR signal(s). The requirement for the plant cuticle in SAR development, the presence of lipids and FAs in petiole exudates (Madey et al. 2002; Behmer et al. 2011; Guelette et al. 2012), and the derivatization of G3P (a glycerolipid precursor) into an unknown compound that translocates with the LTP DIR1, all suggest a role for lipids/FAs/sugars in SAR.

Clearly, more work is required to dissect the relationships between these chemically diverse signals. For example, what factors govern the transport and movement of these signals through the vascular system, and their subsequent unloading into distal tissues? How are these signals processed at their systemic destinations? What reprogramming of metabolic events is required to activate defense and subsequently depress the tissues to the resting phase?

Future Perspectives

The emergence of the tracheophyte-based vascular system had major impacts on the evolution of terrestrial biology, in general, through its role in facilitating the development of plants with increased stature, photosynthetic output, and ability to colonize a greatly expanded range of environmental habitats. Significant insights have been gained concerning the genetic and hormonal networks that co-operate to orchestrate vascular development in the angiosperms, and progress is currently being made for the gymnosperms. However, much remains to be learned in terms of the early molecular events that led to the co-opting of pre-tracheophyte transcription factors and hormone signaling pathways, in order to establish the developmental programs that underlay the emergence of the tracheids as an effective/superior system for water conduction over the WCCs/hydroids. The same situation holds for the FCCs/leptoids to sieve cell/SE transition. Certainly, future application of genomic and molecular tools should offer important insights into the relationships between these pre- and post-tracheophyte/SE programs.

Cost-effective, high-throughput sequencing technologies are opening the door to studies that integrate plant functional genomics with physiology and ecology. Such studies will likely provide important insights into novel strategies, achieved by different plant families, to refine the operational characteristics of their xylem/phloem transport systems to meet the challenges imposed by their specific ecological niches. Much of our current knowledge of vascular development is built upon studies conducted on "model" systems such as *Arabidopsis*. Although the general principles are likely to apply to most, if not all, advanced tracheophytes, many surprises are likely to be unearthed as research expands to cover plants with increased stature and concomitant challenges in terms of environmental inputs.

Fundamental details are now established in terms of the mechanics underlying the thermodynamics of bulk flow though both the xylem and phloem. For the xylem, important

questions remain to be resolved, including the mechanism by which a single cavitation event can propagate within the tracheid/vessel system, the processes involved in refilling of embolized tracheary elements, especially when the transpiration stream is under tension, and the degree to which pit architecture between species contributes to ecological fitness. With regard to the phloem, one of the most fundamental questions that remains to be resolved relates to the mechanism(s) by which the plant integrates sink demand with source capacity to optimize growth under prevailing environmental conditions. The phloem manifold hypothesis and the concept of delivery to various sink tissues being controlled by local PD properties warrants close attention.

The role of the plant vascular system as a long-distance signaling system for integration of abiotic and biotic inputs is also firmly established. However, much remains to be learned concerning the nature of the xylem- and phloem-mobile signals that function in nutrient homeostasis, environmental signaling to control stomatal density in emerging leaves, pathogen-host plant interactions, etc. In addition, the discovery that angiosperm phloem sap collected from the enucleate sieve tube system contains a broad spectrum of proteins and RNA species is consistent with the phloem functioning as a sophisticated communication system. A number of pioneering studies have demonstrated the role of protein and RNA as long-distance signaling agents. Future studies are required to both to expand the number of proteins/RNA investigated as well as to focus on the molecular mechanisms involved in determining how these signaling agents are targeted to specific sink tissues.

Commercial applications of knowledge gained on the development and functions of the plant vascular system are likely to be boundless. Access to methods to control source-sink relationships would have profound effects over yield potential and biomass production for the biofuels industry. Modifications to secondary xylem development will likely allow for engineering of wood that has unique properties for industrial applications. Engineering of novel traits for agriculture will likely be achieved by acquiring a better understanding of the root-to-shoot and shoot-to-root signaling networks. Thus, the future for research on plant vascular biology is very bright indeed!

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