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Preface

The exchange of small solutes and macromolecules between cells is a crucial process for system integration in any multicellular organism. Animals and plants solve the problem of cell-to-cell transport in different ways. In animals transport across the cell membrane is the only, or at least the main, pathway for molecules of different kinds to travel between cells. In plants, however, aside from transmembrane transport, a second (and apparently the most important) transport mode exists, i.e., molecule movement through plasmodesmata—the numerous thin channels connecting living protoplasts. Plasmodesmata allow plant cells to communicate in spite of the cell wall, a more or less rigid layer, surrounding every living plant protoplast. Its presence is responsible for the existence of two different systems—symplasm (protoplasts connected by plasmodesmata) and apoplasm (cell walls and intercellular spaces)—that build every plant organism. Plasmodesmal connections appear as a highly dynamic network, responsible not only for cell-to-cell exchange of organic compounds of a different nature, e.g., carbohydrates and amino acids, but also for movement of signaling macromolecules involved in plant development, such as transcription factors and nucleic acids. Symplasmic transport in plants also regards the movement of solutes and macromolecules over a distance of several meters or even more, by using specialized cells, sieve cells or sieve elements; however, the mechanism of such long-distance transport differs from that of cell-to-cell transport. Hence, symplasmic transport (being responsible for the exchange of solutes and signal macromolecules between cells, tissues, and organs) integrates the plant as the unit.

In the presence of many outstanding papers and books on the processes of transport, the symplasmic transport of molecules in plants seems to have been left aside. In this book we would like to emphasize what an important role symplasmic communication plays in plants. Herein, we would like to concentrate on symplasmic transport of small molecules, although the cell-to-cell transport of macromolecules will also be discussed. We are going to characterize the efficiency of symplasmic transport, mechanisms of molecule passage via plasmodesmata, and the external and internal factors that regulate plasmodesmatal conductivity. In this context, we will concern ourselves with the role of symplasmic domains in plant development, as well as the influence of environmental stresses on the plasmodesmata.

Besides cell-to-cell symplasmic transport, the significance of long-distance symplasmic transport of solutes in phloem elements will likewise be reviewed. We intend to present the mechanism of phloem transport, the processes of symplasmic loading and unloading, as well as the role of pre- and post-phloem transport, with special attention paid to symplasmic transport in wood. Finally, the relevance of the spread of both macromolecules and viruses, via plasmodesmata and phloem, will be presented.

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Chapter 1
Characteristics of Symplasmic Transport

Paweł Sowiński

Abstract Symplasmic transport is possible in organisms of plants, fungi, and even in animals and some prokaryotes, where cell-to-cell protoplasmic junctions are present. However, a spectacular evolution of the symplasm was limited to plants, where highly efficient long-distance transport occurring inside the cells is responsible for the spread of molecules of different nature along the plant body of length up to tens of meters. Several aspects of symplasmic transport are considered in this chapter. A short review of the history of this research is presented with particular attention to old but still inspiring ideas and unanswered questions. Ultrastructure, phylogeny, and ontogeny of the symplasm as well as different mechanisms that allow symplasmic transport (diffusion, cytoplasmic streaming, and mass flow) are discussed thoroughly. Examples of tissues where symplasmic transport covers the distance of several or even more cells without participation of sieve tubes are also discussed, besides the strictly local cell-to-cell symplasmic transport and long-distance transport in phloem.

Keywords Apoplasm • Cytoplasmic streaming • Diffusion • Long-distance transport • Mass flow • Ontogeny • Plasmodesmata • Phloem • Phylogeny • Short-distance transport • Symplasm

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Abbreviations

BS	Bundle sheath
EBS	Extended bundle sheath
KMS	Kranz mesophyll
PCA	Primary carbon assimilation
PCR	Primary carbon reduction
PD	Plasmodesma/plasmodesmata
PVM	Paraveinal mesophyll
SEL	Size exclusion limit
VP	Vascular parenchyma

1.1 Introduction

Life is a flow. Even an immobile single cell demonstrates movement of organelles, vesicles, and cytoplasm streaming. It also exchanges solutes with the environment. The movement of diverse particles and molecular forms is crucial to cooperation between cells in multicellular organisms. To fulfill this demand, a system of cell-to-cell transport has evolved in multicellular organisms. The system comprises protoplasts and cytoplasmic channels bridging neighboring cells—plasmodesmata. They are particularly important for cells enclosed by a cell wall, such as fungi, algae, and plants, but cytoplasmic bridges do connect also animal cells. Plasmodesmata allow not only exchange of small solutes but also of macromolecules such as proteins and nucleic acids, thus forming a versatile system of cell-to-cell communication. The entire system of protoplasts interconnected by plasmodesmata is called the symplasm. It forms the plant body together with the apoplasm comprising cell walls and intercellular spaces. Accordingly, the transport inside and outside cells is called, respectively, symplasmic or apoplasmic. The symplasmic transport system has evolved further in telomic plants parallelly with their increasing size. The corollary was the phloem present in vascular plants. The conducting elements in this system are sieve elements forming sieve tubes transporting phloem sap from leaves to other plant organs. The key feature of this long-distance transport system is that the movement of solutes occurs inside the cells, unlike in the conducting systems functioning in animals, where diverse liquids (e.g., blood, lymph, and food) are transported inside hollow tubes—vessels whose walls are built from cells. Another fundamental difference between those two modes of long-distance transport is that in plants it is not powered by any contracting elements corresponding to the animal heart but solely by hydrostatic gradient along the sieve tubes. Despite its apparent simplicity, the long-distance transport in plants is astonishingly efficient: it allows transport of high amounts of solutes for a distance of several dozen meters in case of some trees. Additionally, it is a pathway for signals of different nature: biochemical, such as

hormones, nucleic acids, and proteins, and biophysical, such as the action and water potentials. This book is focused on the symplasmic transport, but some aspects of the apoplasmic ones will be presented as well.

1.2 Research on the Symplasmic Transport: Milestones

Virtually all reviews on the history of botany begin from Aristotle (384–322 bc); however, the true foundation of modern science is Francis Bacon's (1561–1626) scientific method based on experiment. Probably, the first researchers to contribute substantially to the study of the transport in plants were the inventor of the light microscope, Anton van Leeuwenhoek (1632–1723), who described xylem vessels (after Pardos 2005), and Marcello Malpighi (1628–1694), who showed upstair transport of water in the wood and the downstair transport in bark (after Kursanov 1984). Studies on water movement and transpiration in plants have continued since then (Pardos 2005). The first to study the transport phenomena in plants systematically was Henri-Louis Duhamel du Monceau (1700–1782), considered by many to be the founder of modern plant physiology. At the same time (1774), Bonaventura Corti observed cytoplasmic streaming in plant cells (after Verchot-Lubicz and Goldstein 2010).

According to Zimmermann (1974), intensive studies on transport began in middle of 1800s with the discovery of sieve tubes and of exudation from both phloem and xylem by Theodor Hartig (1805–1880) in 1837. The other ultrastructural component of symplasmic transport, plasmodesmata (PD), was first described by Eduard Tangl (1848–1905), who observed strands of cytoplasm connecting cells in the cotyledon of *Strychnos nux-vomica* (Tangl 1880; after Köhler and Carr 2006a). That discovery attracted soon the interest of numerous investigators (Meeuse 1941). The term *Plasmodesmen* (Germ.) was introduced by Strasburger (1844–1912) in his review (Strasburger 1901). The term "symplasm" was introduced much later by Münch (1930), but yet Tangl noticed that "the connecting ducts unite them (the cells) to an entity of higher order" (after Köhler and Carr 2006b).

Studies at the beginning of the twentieth century added much to the understanding of transport phenomena in plants and, in fact, put forward most of relevant concepts discussed and developed until present. Concerning the plasmodesmata, Meeuse stated in his review (1941) that soon after the Tangl's discovery, the general presence of plasmodesmata throughout the plant kingdom and in all living tissues was accepted. Even animal cells were postulated to contain plasmodesmata (ibid.), but only recently important data in that field have been obtained (Wade et al. 1986; Nicholson 2003; Rustom et al. 2004). The problem of how plasmodesmata develop was discussed already at the beginning of twentieth century, concentrating on the origin of primary (the term not used then) plasmodesmata during the formation of cell walls after mitosis and the secondary (the term used by Meeuse in 1941) ones crossing existing cell walls. At those primordial stages of research on

plasmodesmata, their role in the cell-to-cell transport as well as the origin of sieve pores from plasmodesmata were first postulated. Although there was much concern regarding possible artifacts of specimen fixation, the protoplasmic nature of the plasmodesmata was generally accepted. The most convincing piece of evidence was that "there is a translocation of viruses from cell to cell" (ibid.), a phenomenon being the research area studied to date.

In the case of long-distance transport, most of the early studies concentrated on the ascent transport of water and some modern ideas on phloem transport were formulated as well. In particular, Dixon, who proposed the cohesion theory of water transport above the barometric height (Dixon 1914), neglected diffusion as a mechanism of transport of organic compounds from leaves to other organs (Dixon and Ball 1922). Those authors calculated that, even if the transport of sucrose solution was accelerated by protoplasmic streaming, the diffusion rate was too low to account for the actual rate of transport of carbohydrates in plants. Later on Münch (1930) formulated his concept of pressure flow, fully accepted only recently, to explain the mechanism of long-distance transport. He also coined the terms "symplasm" and "apoplasm."

Any further progress in the research on the symplasmic path components was crucially dependent on the developments of experimental techniques. Systematic studies were performed on the mechanism of cytoplasmic streaming (Kamiya and Kuroda 1956; Kamiya 1981 and citations therein). At the same time, the velocity of organelles' movement was measured at 2–5 μm s^{-1} and 5–6 μm s^{-1} for chloroplasts and vesicles, respectively (Zubrzycki 1951). One cannot but admire the accuracy of those estimates obtained with rather crude tools: the most recent measurement of the velocity of small organelles using GFP fused to peroxisome targeting signal 1 (PTS1) and time-lapse laser scanning confocal microscopy reported an almost identical value of 10 μm s^{-1} (Jedd and Chua 2002).

Regarding the mechanism of long-distance phloem transport, several hypotheses were formulated besides the pressure flow theory of Münch, even though the finding of Mittler (1957) that the turgor pressure in sieve elements was high enough to explain the observed velocity of phloem transport spoke eloquently in favor of the latter. Those challenging the Münch theory claimed that sieve pores were always occluded by a dense material which precluded an efficient mass flow of solutes under pressure. On the basis of that observation, Spanner proposed his concept of electroosmosis (Spanner 1975) as an alternative for the pressure flow theory. It was argued that if sieve pores were indeed narrowed by occludes, electrical phenomena would be more efficient in powering of the phloem transport than pressure flow. In several other hypotheses, described in reviews of Canny (1975) and Kursanov (1984), pulsations of microstructures in the sieve tubes were proposed as the motive force for transport. Another proposition was the movement in monolayer, i.e., sliding of molecules along the phase boundaries due to uneven distribution of molecular forces (ibid.). Additionally, in many electron microscopic studies, P-proteins were found forming strands along the sieve tubes. These observations prompted hypotheses on the participation of P-proteins in longitudinal transport despite their scarcity or even absence in many plants, e.g., maize and

barley (Evans 1976). One of them proposed P-proteins as ducts for electric waves powering the longitudinal transport in sieve tubes (Hejnowicz 1970). In the 1980s and 1900s, most of those hypotheses were discredited as either unrealistic or based on artifacts.

The final argument for the Münch's pressure flow was found at the very end of the twentieth century, when Ehlers et al. (2000) showed that carefully fixed sieve tubes did not show any occlusions at the sieve pores and the lumen of sieve tubes was clear. It seems that most of the artifacts found in sieve tube preparations were related to the sample preparation (ibid. and references there) and induction of mechanisms preventing the leakage of the phloem sap from injured sieve tube. The response seems to be particularly sensitive (Knoblauch and van Bel 1998) to even delicate mechanical stress.

Currently, the pressure flow theory of long-distance transport in the phloem seems to be widely accepted. Nevertheless, three ideas concerning phloem transport formulated in the twentieth century, outside the mainstream considerations in this area, seem worth mentioning.

The first question concerns the problem of bidirectional transport in the phloem. The movement of different molecules in opposite directions in individual sieve tubes was assumed as a strong argument against mass flow mechanism (for reviews, see Evans 1976; Kursanov 1984). It is, however, possible that phloem transport in opposite directions occurs in separate sieve tubes. Additionally, modeling the dynamics of solute transport (Henton et al. 2002) has demonstrated that the solute can move in opposite directions along the single tube. No experimental current data on the problem are accessible, beside of technical progress and development of methods.

The second problem was formulated by Romberger et al. (1993) and concerned the mechanism of phloem transport. Basing on their calculations, those authors stated that the sieve pores were too wide for efficient electroosmosis, which neglected Spanner's electroosmosis theory, yet they were too narrow for efficient mass flow of solutes which excluded the Münch's pressure flow theory. In conclusion, the authors proposed that if pressure flow was accelerated by electroosmosis, the pore diameter would be optimal for the transport. The concept has not been developed further.

The third idea concerns the so-called vacuome, i.e., a system of connections between vacuoles crossing the plasmodesmata as desmotubules and involving also the sieve tube lumen (Gamalei and Pakhomova 2002; Velikanov et al. 2005). Already Esau (1971) reviewed the suggestions of several researchers that a membrane (tonoplast) could separate the parietal cytoplasm from the central cavity in sieve tubes. Against such a hypothesis were reports describing the disappearance of the tonoplast in mature sieve elements (for review, see Kursanov 1984), although other authors argued for an extreme sensitivity of tonoplast to preparation and fixation (Esau 1971). Contacts between vacuoles and plasmodesmata were shown by some authors. Rinne et al. (2001) demonstrated that spherosome-like vacuoles became displaced toward plasmalemma near plasmodesmata during the releasing from dormancy in the apical meristems of *Betula pubescens*. The postulated role of

the movement was limited to the transient delivery of β-1,3-glucanase to the plasmodesmata. A vacuolar-tubular continuum was also reported in trichomes of *Cicer arietinum* (Lazzaro and Thomson 1996). The idea of participation of vacuome in assimilate transport from mesophyll chloroplasts to sieve tubes and then to other organs, presented by Gamalei (2007), is based almost exclusively on the results of Gamalei, and his collaborators and therefore a critical discussion by others would be desirable to add credence to it.

1.3 Ultrastructure, Ontogeny, and Phylogeny of Symplasm

In higher plants, the symplasm consists of protoplasts linked by plasmodesmata and sieve elements involved, respectively, in cell-to-cell and long-distance transport. The ultrastructural details of plasmodesmata and sieve tubes are discussed in other chapters of this book. Therefore, only basic information is provided here.

1.3.1 Ultrastructure and Ontogeny

1.3.1.1 Plasmodesmata

Plasmodesmata in higher plants are cytoplasmic channels penetrating cell walls with the plasmalemma as the outer border and a desmotubule in the center inside the channel. The diameter of plasmodesmata is 20–50 nm (Ehlers and Kollmann 2001), and their length reflects the cell wall thickness. However, depending on the tissue and developmental stage, plasmodesmata may differ strongly in shape and form (Robinson-Beers and Evert 1991; Ehlers and Kollmann 2001; Botha 2005; Burch-Smith et al. 2011). They can be simple or branched, with or without constrictions in the neck and/or central regions. The desmotubule is approximately 15 nm in diameter (Ehlers and Kollmann 2001), occupies the center of the plasmodesma channel, and is a tubular process of the endoplasmic reticulum membrane connecting the ER systems of the neighboring cells. It is used to be also called the "central rod" due to its apparently solid structure on most of plasmodesmata microphotographs (Gunning and Overall 1983; Tilney et al. 1991; Botha et al. 1993; Overall and Blackman 1996; Ding 1998). Recent data show, however, that the desmotubule is a membranous tubule composed of lipids and proteins, the latter allowing an extremely strong contraction of the tube (Tilsner et al. 2011). The desmotubule is surrounded by a cytoplasmic sleeve penetrated by spoke-like proteinaceous extensions linking the desmotubule with the plasmalemma. In principle, the transport routes through plasmodesmata could involve the cytoplasmic sleeve, the desmotubule membrane, and the desmotubule lumen (Evert et al. 1977; Waigmann et al. 1997; Cantrill et al. 1999; Roberts and Oparka 2003; Sowiński et al. 2008; Barton et al. 2011), but only the first one is widely accepted in the literature.

Two main types of plasmodesmata are discussed in the literature: primary and secondary ones (Ehlers and Kollmann 2001; Burch-Smith et al. 2011). It is widely accepted that primary plasmodesmata are those forming during cell division. The case of secondary plasmodesmata is less clear-cut, since to some authors secondary PD are only those formed across preexisting cell walls, while others use the term also for PD formed by modification of primary PD, such as PD twinning. To avoid misunderstanding, some authors speak of "twinned secondary plasmodesmata" and "de novo secondary plasmodesmata" (Burch-Smith et al. 2011).

In general, plasmodesmata can undergo distinct modifications during plant development. Between some cells, plasmodesmata can be eliminated, which leads to symplasmic isolation of the symplasmic domains (Rinne and van der Schoot 2003), an important step in plant development (see Chap. 2). At other locations, the plasmodesmata become branched at certain developmental stages, thereby providing an improved symplasmic transport path; examples are the plasmodesmata linking intermediary cells (a form of companion cells) with sieve elements in symplasmic phloem loaders (Volk et al. 1996, see Chap. 5). A unique plasmodesma modification is its conversion into a sieve pore, occurring during the development of sieve elements (Sjölund 1997). This modification involves widening of the pores up to 200–400 nm, thus allowing almost unimpeded symplasmic transport in sieve tubes by means of the pressure flow mechanism.

1.3.1.2 Phloem

Mature sieve tubes in angiosperms are columns of elongated cells, sieve elements, up to 20 µm in diameter and 250 µm in length (Sjölund 1997). They contact one another in the file through sieve plates massively penetrated by sieve pores. The sieve elements contain no nucleus, vacuoles, ribosomes, Golgi bodies, microfibrils, or microtubules (van Bel and Knoblauch 2000). Their only structural components are the modified ER, mitochondria, and plastids (ibid.). The latter are either P-plastids or S-plastids, containing proteinaceous or starch inclusions, respectively (ibid.). The presence of P-plastids and S-plastids is family specific (Behnke 1991). The elimination of the other organelles occurs during sieve element maturation and is accompanied by cell wall thickening and conversion of "ordinary" plastids into the S- or P-plastids. The cytoplasm is apparently present only at the cell periphery with the organelles linked to the plasmalemma by clamps (Ehlers et al. 2000). Thus, the central part of the sieve element is empty, allowing efficient transport of the phloem sap.

The sieve elements are joined to companion cells, both structurally (numerous plasmodesmata branching at the companion cell side) and functionally (the companion cells provide proteins for the sieve elements and may also participate in phloem loading; see Chap. 5). Thus, the cells of both types are often treated as a unit: a companion cell/sieve element complex. Besides their structural and functional cooperation, both sieve elements and companion cells originate from phloem mother cells; one sieve element and several companion cells form one mother cell (van Bel 2003), which differentiates them from the sieve cells of other vascular plants.

Table 1.1 Symplasmic pathway in prokaryotes and animals

		Plasmodesmata		Conducting cells	
Kingdom	Phylum	Structural aspects	Functional aspects	Structural aspects	Functional aspects
Eubacteria	Cyanophyta	Microplasmodesmata	Rapid intra-filament movement of metabo-lites, possibly transfer of signals	No special-ized cells regarded	
Animalia		Gap junctions, <4 nm in diameter, composed of connexins (vertebrates) or innexins (invertebrates)	Transport of solutes of low molecular weight (<1.7 kDa)		
		Tunneling nanotubes (found in mammals) cytoplasmic channels (possible transient), 50–200 nm in diameter and length of tens of μm, enriched with actin filaments but lacking microtubules	Transfer of endosome-based vesicles, organelles, viruses	No special-ized cells regarded	

1.3.2 Phylogeny

For decades, a dogma was in force that the symplasm and symplasmic transport were limited to plants and some fungi. The challenge of this dogma has come only recently with the discovery of tunneling nanotubes (Rustom et al. 2004) in animals, although even much before that gap junctions linking animal cells were widely known (Wade et al. 1986; Nicholson 2003), but their existence seemed not to violate the view of the animal tissue as a sum of isolated cells exchanging "signals" and molecules only via specialized relay and channel proteins. Nowadays, it is commonly accepted that all multicellular organisms demonstrate direct cell connections (Baluška et al. 2004; Gerdes et al. 2007; Rustom 2009), albeit the extent of such "symplasmic" network varies greatly among kingdoms.

The current state of our understanding of the symplasmic connection at the cell and organismal levels is summarized in Tables 1.1, 1.2, 1.3, 1.4, 1.5, and 1.6, compiling of data from many original papers, reviews, and monographs concerning animals (Wade et al. 1986; Nicholson 2003; Rustom et al. 2004), fungi (Kirk and Sinclair 1966; Powell 1974; Marchant 1976; Taylor and Fuller 1980; Cook and Graham 1999; Müller et al. 1999), nonvascular plants (Schmitz and Srivastava

Table 1.2 Symplasmic pathway in fungi

		Plasmodesmata		Conducting cells	
Kingdom	Phylum	Structural aspects	Functional aspects	Structural aspects	Functional aspects
Fungi	Basidiomycota	Simple plasmodesmata		Central pore in septa	Cytoplasmic streaming, communication between cells, flow of cytoplasm and organelles, dead cells in hypha isolated by plugging of the central pore
	Ascomycota	Simple PD, 60 nm in diameter with desmotubule (10 nm in diameter) linked to ER		Central pore in septa	Cytoplasmic streaming, communication between cells, flow of cytoplasm and organelles
	Chytridiomycota	Simple PD (23 nm in diameter) with dense core			
	Zygomycota	Simple PD linked to ER			

1974; Marchant 1976; Cook et al. 1997; Cook and Graham 1999; Kwiatkowska 1999, 2003), lower vascular plants (Mueller 1972; Evert and Eichhorn 1976; Warmbrodt and Evert 1979; Smoot 1985; Evert et al. 1989; van Bel 1999; Cooke et al. 2000; Raven 2003; Dong et al. 2004; Halarewicz and Gabryś 2012), and higher vascular plants (Glockmann and Kollmann 1996; Cook and Graham 1999; van Bel 1999; Beck 2010).

1.3.2.1 Plasmodesmata

The lowest common denominator of the symplasmic contacts in all eukaryonts and some prokaryotes is cytoplasmic channel of 20–30 nm in diameter, with the exception of narrower gap junctions in animals and microplasmodesmata in *Cyanophyta* (Table 1.1). Since the discovery of tunneling nanotubes is fairly recent, comprehensive study is still to come. Only then will comparative analysis of cell-to-cell connections throughout the kingdoms become meaningful. For now, one may conclude that the appearance of complex structures in the channels connecting cells is unique to the plants. The desmotubule and the ability to form de novo secondary plasmodesmata are of particular interest here.

Table 1.3 Symplasmic pathway in algae

		Plasmodesmata		Conducting cells	
Kingdom	Phylum	Structural aspects	Functional aspects	Structural aspects	Functional aspects
Plantae	Rhodophyta			Elongated cells with cross walls with pit plugs	Possibly mass flow
	Phaeophyta	Simple PD, some authors report desmotubules		Conducting filaments, length of 1 mm and more, numerous pores in cross walls, small vacuoles, many mitochondria and plastids, nucleus degenerates at maturity, ER, ribosomes, and coated vesicles	Possible mass flow, assimilate transport, accumulation of callose with age and degeneration of some organelles
	Chlorophyta	Simple PD, some authors report ER inside plasmodesma		Giant cells, intercellular connection by PD	Cytoplasmic streaming by means of actin-myosin mechanism
	Charophyta	Simple PD with uncompressed ER inside at some developmental stages; possible secondary PD	Plugging or disappearance at some developmental stages	Giant cells, intercellular connection by PD	Cytoplasmic streaming by means of actin-myosin mechanism

Table 1.4 Symplasmic pathway in lower vascular plants

		Plasmodesmata		Conducting cells	
Kingdom	Phylum	Structural aspects	Functional aspects	Structural aspects	Functional aspects
Plantae	Marchantiophyta	Simple PD with desmotubules		Elongated cells in gametophyte, vacuoles disappeared at maturity, numerous PD in end walls, ER, other organelles present. Absent in sporophytes	Cell polarization
	Anthocerotophyta	Simple PD with desmotubules		Absent in gametophytes and sporophytes	
	Bryophyta	Cytoplasmic bridges, similar to PD, no desmotubule. Single or branched plasmodesmata with desmotubules	Possible role during spermatogenesis	Conductive parenchyma cells; in *Polytrichaceae*: leptoides in gametophytes, dimension 500 × 20 μm, frequent PD of 120–250 nm in diameter in end walls, nucleus degradation, lack of large vacuoles, ER, different organelles. In sporophyte absent or similar to those in gametophyte	Cell polarization

Table 1.5 Symplasmic pathway in lower vascular plants

		Plasmodesmata		Conducting cells	
Kingdom	Phylum	Structural aspects	Functional aspects	Structural aspects	Functional aspects
Plantae	Lycopodiophyta	Primary PD		Long, narrow sieve cells, nuclear remnants, no vacuole, mitochondria and plastids, tubular network of ER, no difference between pore sizes in end and lateral walls	Adjoining parenchyma cells linked by numerous PD
	Polypodiophyta	Primary PD		Sieve cells with nuclear remnants, no vacuole, smooth ER, mitochondria, lack of plastid grana in some species, no difference between pore sizes in end and lateral walls, considerable variation in the size of cytoplasmic connections among different fern species (0.06–0.7 μm)	Adjoining parenchyma cells linked by numerous PD

The desmotubule was reported as a plasmodesma constituent for vascular plants (Cook et al. 1997; Table 1.4). However, some authors reported the presence of ER protrusions into plasmodesmata in the less evolutionarily advanced plants, *Phaeophyta, Chlorophyta,* and *Charophyta* (Cook and Graham 1999; Table 1.3). Interestingly, desmotubule was found in one division of the fungi, the *Ascomycota* (Marchant 1976; Table 1.2). It is, however, generally accepted that the desmotubule is an "invention" of vascular plants; similarly, the ability to form de novo secondary plasmodesmata seems to have developed in higher plants (Table 1.6). Cooke et al. (2000) went even as far as to propose that this trait is limited to angiosperms. However, one should note two reports (Wetherbee et al. 1984; Franceschi et al. 1994) that some *Charophyta* and *Rhodophyta* divisions are also able to form secondary plasmodesmata. For a thorough discussion on secondary plasmodesmata formation in nonvascular plants, the reader is referred to the review of Kwiatkowska (1999). Although over a decade old, it is, to my knowledge, the most recent comprehensive analysis on the subject. Also, one should note that secondary plasmodesmata are formed in gymnosperms, at least in conifers. Bearing in mind that in conifers the sieve cells and Strasburger cells do not originate from common mother cells as do sieve elements and companion cells in angiosperms (van Bel 1999), plasmodesmata linking the sieve cells and the Strasburger cells should by definition

Table 1.6 Symplasmic pathway in higher vascular plants

	Plasmodesmata		Conducting cells	
Phylum	Structural aspects	Functional aspects	Structural aspects	Functional aspects
Pinophyta	Simple or branched PD with desmotubules		Very long sieve cells with overlapping ends, nuclear remnants, modified plastids, smooth ER, cell components located peripherally	Strasburger (albuminous) cells = modified parenchymatic cells (no true companion cells)
Magnoliophyta	Simple or branched PD, primary or secondary with desmotubules	Transport of low molecular weight solutes (<1 kDa), macromolecules (proteins, RNA); transfer of viruses, viroids; possible transfer of whole genomes of mitochondria and chloroplasts	Sieve elements, no nucleus, modified plastids, some mitochondria, smooth ER, cell components located peripherally, sieve pores in transverse walls, branched PD at companion cell side	True companion cells (same cell line as sieve elements). In grasses, two forms of sieve elements: thin walled (similar to "obscure" sieve elements, connected to companion cells) participating in long-distance transport; thick walled of unknown function, lacking of companion cells, linked by PD to adjoining parenchyma cell

be treated as secondary ones. Additionally, since some conifer species can be grafted, one should expect formation of secondary plasmodesmata between cells of the scion and stock.

1.3.2.2 Conducting Elements

A spectacular evolution of the symplasm was limited to plants. It involved elongation of some cells and their specialization in transport of organic molecules at longer distances. Actually, also in the fungi divisions of *Ascomycota* and *Basidiomycota*, where cells are separated by septa with septal central pores, a sort of long-distance transport driven by cytoplasmic streaming (as in green algae) may function since septal pore allows movement of cytoplasm and organelles (Müller et al. 1999). The septal pores resemble the pit plugs of red algae, apparently an example of convergent evolution (Cook and Graham 1999). The symplasmic transport system developed fully in vascular plants (Table 1.6). The increase of the size of land plants according to the Cope's rule stating that the average body size in a population tends to increase over evolutionary time (after Enquist 2003) puts a strong selection pressure on the development of efficient long-distance transport systems. In the case of xylem, selection resulted in minimization of resistance within xylem vessels (ibid.) by removing all their contents and widening the pores in transverse cell walls. Apparently, the same concerned the sieve cells, since the general evolutionary trend here was the elimination of organelles and cytoplasm content and the formation of the sieve pores in transverse cell walls. Despite these similarities, a crucial difference between xylem vessels and sieve cells remained, since the latter are live cells which greatly increases the resistance to flow. The reason for that could be that the transport in sieve tubes is often against the air-soil water potential gradient; therefore, a positive water pressure must be imposed on the water column in the pipe. Another plausible reason is that only living cells can offer efficient mechanisms of defense against leakage of the precious in case of wounding or herbivore attack. Indeed, another trend in the evolution of sieve cells was the development of such defense mechanisms, e.g., callose accumulation at the sieve plate and other mechanisms of sieve cells sealing.

1.4 What, How, and How Fast: Mechanisms and Efficiency of Symplasmic Transport

1.4.1 What Is Transported in Symplasm?

Myriads of molecules are transported every second in a living plant. Roughly, they can be divided into the low molecular weight and high molecular weight ones. Inorganic ions and organic compounds of different nature of a mass below ca. 1 kDa belong to the first class, while molecules of several kDa and heavier belong to the

second one. In general, the transporting limit of plasmodesmata is called size exclusion limit (SEL), which usually is ca. 900 Da (Crawford and Zambryski 1999). Actually, the better measure is a molecule's Stokes radius, i.e., the radius of a sphere whose hydrodynamic properties mimic those of the molecule. Some other restrictions are related to the physicochemical properties of the molecules to be transported. Thus, several types of molecules are not transported through plasmodesmata, like auxins (Drake and Carr 1978), small hydrophilic molecules of charge in range −4 to −2; molecules containing either Phe, Try, Met, or His groups (Tucker and Tucker 1993); and aromatic amino acids (Tucker 1982; Erwee and Goodwin 1984). One should note, however, some discrepancies concerning the transport of aromatic amino acids either polar or hydrophobic types (Terry and Robards 1987). Possible, plasmodesmata from different tissues differ in their properties (see below, Sect. 1.5.1).

SEL may change under some circumstances to allow transport of high molecular weight molecules through plasmodesmata. Proteins and nucleic acids, as well as bigger complexes, as viruses are now known to be transported in plants through plasmodesmata (Lucas et al. 2009; Maule et al. 2011; Xu and Jackson 2010). For details the reader is referred to Chap. 7. Here, however, it is worth noting that even whole organelles seem to be transported in symplasm, at least through plasmodesmata. Recently, two laboratories independently have presented evidence for the cell-to-cell movement of an entire plastid genome from one plant to another, possibly in an intact organelle (Stegemann et al. 2012; Thyssen et al. 2012). This finding explains former observations of horizontal gene transfer between stock and graft in some grafting experiments (Stegemann and Bock 2009; Talianova and Janousek 2011). Transfer of entire organelles between cells is possible in fungi, nonvascular plants, and lower vascular plants (Tables 1.2, 1.3, and 1.4). The possibility of movement of plastids and mitochondria opens a new area of research on the mechanism of transport of such big organelles. The finding is also intriguing in the context of discussion on the role of horizontal gene transfer in plant evolution as well as its consequences for the safety of GMO in environment.

In the phloem, sucrose and other oligosaccharides are main solutes at concentrations depending on the phloem loading mode (see Chap. 5), while reducing carbohydrates, such as fructose, are absent (Kursanov 1984). Besides carbohydrates, amino acids produced in leaves are transported from leaves to other organs as well (see Chap. 6). The phloem sap also contains inorganic ions at pretty high concentrations, except magnesium, calcium, and boron, which are practically absent (Marschner et al. 1996; Brown and Hu 1996; Atkins 1999). It is worth noting that also heavy metal ions of no apparent use to the plant can translocate through the phloem (for review, see Antosiewicz et al. 2008). Phloem sap contains many other endogenous substances, e.g., secondary metabolites of diverse biological activities such as alkaloids (Kitamura et al. 1993), glucosinolates (Brudenell et al. 1999; Chen et al. 2001), hormones (gibberellins) (Hoad et al. 1993), ABA (Zhong et al. 1996), and precursor of ethylene ACC (Morris and Larcombe 1995). Also auxins and cytokinins are detected in the phloem sap at concentrations sufficient for their biological activity (Baker 2000a, b), even though auxins are generally transported in the cell-to-cell manner outside the vascular systems and cytokinins are synthesized in roots. Also diverse important constituents of the phloem sap are proteins and nucleic acids.

Apart from endogenous substances, phloem translocates also foreign molecules of neutral or negative influence on plant, such as many xenobiotics of herbicide activity (for review, see Brudenell et al. 1995). They are loaded into phloem by carrier-mediated mechanism (Delétage-Grandon et al. 2001) or by diffusion through plasmalemma depending on the physicochemical and structural properties of a given herbicide. Thus, e.g., the non-ionized, monobasic weak acids diffuse easily (Bromilow and Chamberlain 2000). Phloem is also the route of dispersal of viroids (Palukaitis 1987; Zhu et al. 2002), viruses (Esau 1956; Lucas 2006), and bacteria (Rudzińska-Langwald and Kamińska 1999; Moran 2001). The illustration of bacteria presence in sieve tubes is shown on Fig. 1.1. Thus, in addition to its crucial role in the transport and distribution of photosynthates and in systemic signaling, phloem is also plant's Achilles' heel, since it allows invasion by pathogens as well as facilitates the action of herbicides.

1.4.2 Mechanisms of Symplasmic Transport

As already mentioned, most of the transport in plants take place in the symplasm. Three main transport mechanisms have been proposed to explain the movement of molecules in the symplasm: diffusion, cytoplasmic streaming, and mass (bulk) flow. Diffusion is a motion of molecules in a solvent (fluid, gas) according to concentration gradient and is required for numerous cellular processes (Verkman 2002). Cytoplasmic streaming has been classified in physical terms as a form of convection (Pickard 2003), i.e., a movement of distinct zones of fluid. However, the term "advection" seems to be more proper for cytoplasmic streaming (Verchot-Lubicz and Goldstein 2010). Advection is a transport mechanism of a dispersed substance (molecules, particles, etc.) by a fluid due to the fluid's bulk motion. Formally, the term convection is used to refer to the sum of advective and diffusive transfer. The third mechanism could be defined as the movement of a solute molecule together with solvent molecules according to the gradient of a physical force (e.g., water pressure gradient). All three mechanisms have been the subject of numerous, both experimental and theoretical, modeling.

1.4.2.1 Diffusion

Diffusion is assumed to be an important mechanism for intra- and intercellular movement of solutes. According to Tucker (1990), cell and plasmodesmata should be treated as separate diffusion systems. As measured *in vivo*, the velocity of diffusion through plasmodesmata is in the range of 2.8–17 μm s^{-1} (after Anisimov and Egorov 2002), while between cells in a file only 1.1–8.5 μm s^{-1} (Rutschow et al. 2011 and citations there). The diffusion coefficient calculated for plasmodesmata (ibid.) was 2–20 times higher than across the corresponding cell walls (Kramer et al. 2007). Both studies measured the transport of carboxyfluorescein

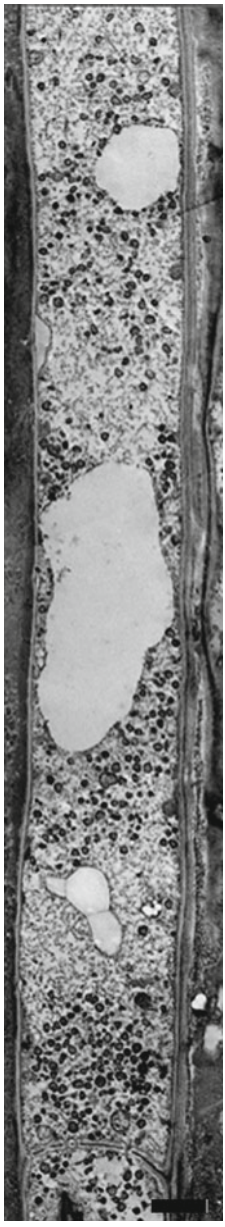

Fig. 1.1 Bacteria (spheroidal structures) in immature sieve tubes of shoot apex of *Catharanthus roseus* plants, bar = 2μm (Courtesy of Dr. Anna Rudzińska-Langwald, Department of Botany, Faculty of Agriculture, Warsaw Agricultural University)

(CF) in *Arabidopsis* roots. Additionally, apoplasmic transport may be limited by the kinetic properties of a given carrier (affinity, capacity). A comparison of rates of symplasmic and apoplasmic modes of transport (Patrick and Offler 1996) showed an even bigger advantage of symplasmic transport, possibly related to a membrane transport component. Hence, symplasmic transport by means of

diffusion between cells seems to be the most efficient mode of transport. Some authors postulated, however, that transport through plasmodesmata occurs by means of mass flow (Patrick and Offler 1996; Voitsekhovskaja et al. 2006) involving a parallel flow of solvent and solutes. This would require permanent flow of water through plasmodesmata.

Diffusion has some limitations as a mechanism of solute movement in the cytoplasm. They are related to fluid-phase viscosity, interactions of the solute molecules with other components of the cytoplasm, and the resistance of the diffusion medium due to accumulation of intracellular components, i.e., molecular crowding (Verkman 2002). The effect of fluid-phase viscosity on solute diffusion in the cytoplasm is neglected by some authors (Fushimi and Verkman 1991; Luby-Phelps et al. 1993) showing experimentally that the viscosity of the cytoplasm is just 10–30 % higher than that of water. On the other hand, *in vivo* measurements of GFP (27 kDa) movement in *Escherichia coli* cells found cytoplasm ten times more viscous than water (Sear 2005). Also the binding of solute molecules to other components of the cytoplasm seems to have little impact on diffusion (Verkman 2002); one should note, however, that this conclusion was arrived at the basis of measurements for a fluorescent probe; BCECF and the binding effect for endogenous molecules of different character could be more pronounced. The strongest negative effect on diffusion was related to the crowding, for both small solutes and macromolecules (ibid.). Some other limitations for diffusion as the means of solute transport in the cytoplasm came from theoretical considerations regarding the nature of diffusion (Pickard 2003). An example is the randomness of diffusion which makes directional transport of rare metabolites highly inefficient (ibid.). Also diffusion through plasmodesmata has its limits (Sowiński et al. 2008) due to the transport channels being not much wider than the Stokes radii of the solutes (Cui 2005; Liu et al. 2004), the tortuosity of these channels (Malek and Coppens 2003), and binding of solutes by the channel walls (Cui 2005; Valiullin et al. 2004). The constraints to molecule diffusion in plasmodesmata are shown on Fig. 1.2.

One should note, however, that our knowledge on the architecture of such delicate structures as plasmodesmata seems to be far from complete, so the modeling of solute flow along these structures can only suggest new research approaches rather than unequivocally explain the mechanism of flow transport.

1.4.2.2 Cytoplasmic Streaming

Most of the aforementioned problems concerning diffusion in the cytoplasm do not apply if one considers the role of cytoplasmic streaming in moving the cytoplasm components (Pickard 2003). Cytoplasmic streaming takes different shapes: unidirectional, circular, fountain, or rotational (Fig. 1.3). The velocity of cytoplasmic streaming was studied mostly in the alga *Chara* sp. because of its large cells. The use of different methods including direct observations under light microscope (Kamiya 1959) and modern, sophisticated methods as laser-Doppler velocimetry (Ackers et al. 1994) and magnetic resonance velocimetry (van de Meent et al. 2010)

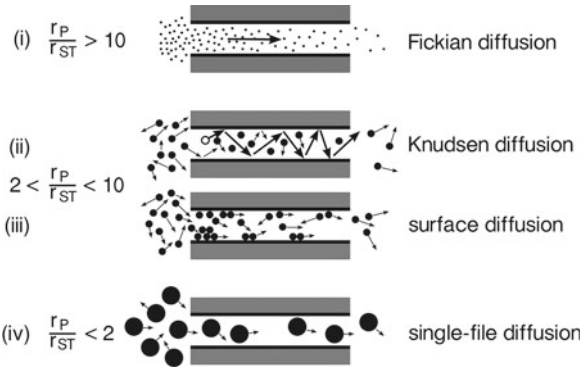

Fig. 1.2 Types of diffusion in narrow tubes. (i) Fickian diffusion (no constriction to diffusion), pore diameter is over 10 times more than that of transported molecule, (ii) Knudsen's diffusion (constriction to diffusion due to molecule collisions with pore walls), (iii) surface diffusion (constriction to diffusion due to molecule adhesion with pore walls), pore to molecule diameter 2–10, and (iv) single-file diffusion (the strongest constriction to diffusion), pore diameter smaller than two molecular diameters. r_p pore radius, r_{ST} Stoke's radius of transported molecule (Courtesy of Dr. Jarosław Szczepanik, Department of Plant Molecular Ecophysiology, Institute of Plant Experimental Biology and Biotechnology, Faculty of Biology, University of Warsaw)

showed that the velocity of cytoplasmic streaming in internodal cells is rather high, in the range of 40–100 μm s^{-1}. A similar high speed of cytoplasmic streaming was also observed in the plasmodia of myxomycetes, another classic material for studying of the phenomenon (Kamiya 1981). In contrast, the velocity of cytoplasmic streaming in higher plants was reported as severalfold lower than in *Charophyta* (Kamiya 1959).

Cytoplasmic streaming seems to be driven indirectly by organelles and diverse vesicles bound to myosin sliding along actin microfilaments (Shimmen and Yokota 2004). The streaming of the cytoplasm speeds up distribution of solutes along the cell immensely. In a recent review on the topic (Verchot-Lubicz and Goldstein 2010), the authors noticed that fluid would move across a giant *Chara* cell in 17 min by means of cytoplasmic streaming but would require almost 3 months to achieve it by diffusion. Cytoplasmic streaming as a transport mechanism has its own limitations, however. Since cytoplasm is a medium of rather high viscosity and low Reynolds number, laminar flow takes place instead of turbulent flow typical for media of high Reynolds numbers. A consequence of laminar flow is little mixing of the medium layers. Thus, for efficient pole-to-pole delivery of solutes and vesicles in the cell, cytoplasmic streaming and diffusion mechanisms should work in concert.

Cytoplasmic streaming is commonly assumed to be limited to intracellular transport. One should note, however, that it could also participate in long-distance transport in some organisms (Tables 1.2 and 1.3), i.e., *Basidiomycota, Ascomycota* fungi, and *Charophyta* and *Chlorophyta* algae. In these organisms, cytoplasmic streaming

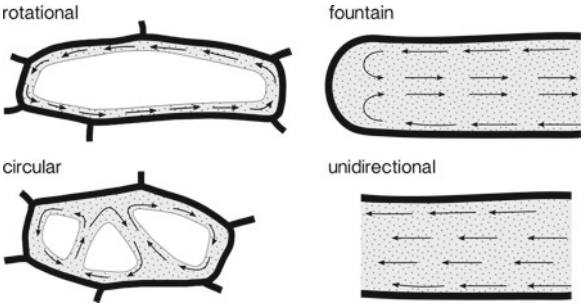

Fig. 1.3 Different shapes of cytoplasmic streaming. Rotational and circular cytoplasmic streaming typical for plant cells. Fountain and unidirectional cytoplasmic streaming typical for fungi hyphae (Courtesy of Dr. Jarosław Szczepanik, Department of Plant Molecular Ecophysiology, Institute of Plant Experimental Biology and Biotechnology, Faculty of Biology, University of Warsaw)

may participate in long-distance transport of solutes, vesicles, and even organelles. In higher plants, cytoplasmic streaming was postulated to occur also in the phloem (Jedd and Chua 2002) to explain the observed movement in sieve tubes. However, the mechanism of such a movement would be difficult to conceive since sieve elements lack a cytoskeleton (van Bel and Knoblauch 2000).

1.4.2.3 Mass Flow

The phloem translocation in higher vascular plants is highly effective. The high velocity of phloem transport has fascinated researchers since the rise of plant physiology. Diverse methods were used to measure it, including calculation of linear speed based on estimates of mass transfer between source and sink (Zimmermann 1974) or the rate of phloem sap leakage from sieve tubes (Mittler 1957), direct *in vivo* measurements of transport speed of photosynthates labeled with radioactive carbon isotopes, ^{11}C (Fensom et al. 1977; Magnuson et al. 1982; Jahnke et al. 1998) or ^{14}C (Sowiński et al. 1990, 2007; Black et al. 2012), as well as of the water flow along phloem measured by means of NMR (Rokitta et al. 1999). The velocity of phloem transport may reach 1 m h^{-1} (ca., 300 μm s^{-1}) or even more. Thus, it exceeds maximal values of velocity of cytoplasmic streaming (up to 100 μm s^{-1}) and diffusion (up to 10 μm s^{-1}) 3 and 30 times, respectively. To underline the efficiency of phloem translocation, Sjölund (1997) has expressively compared the phloem sap flow in sieve tubes to a river of 20 m wide flowing at speed 400 km h^{-1}. However, a comparison of sieve tubes with animal blood vessels of a similar diameter seems more informative. Velocity of blood flow in conjunctival vessels 20–50 μm in diameter was 300–1,500 μm s^{-1} (Mayrovitz et al. 1981), i.e., very close to the linear speed of translocation in 20 μm sieve tubes. Thus, despite the distinct mechanism that drives the fluid in sieve tubes of higher plants (no mechanical pumps) as well as the different nature of the vein (transport inside the cell files), the efficiency of the

transport system in supporting sink organs often tens of meters away from the source leaf with photosynthates and other organic compounds is as high as in vertebrates; a highly effective system of transporting molecules throughout the body was pivotal in allowing plants to colonize land.

The long-distance transport in the phloem is driven by pressure flow. This simple statement by Münch (1930) used to be challenged periodically by many during the last 100 years (see above, Sect. 1.2) but has finally been accepted. Pressure flow is the effect of a water pressure gradient between the source (adult leaves in most cases) and sinks (other organs), caused mainly by two processes, phloem loading in collection phloem and phloem unloading in release phloem, the former responsible for the delivery of carbohydrates into and the latter out of sieve tubes. Carbohydrates are osmotically active; thus, in the phloem loading zone, water is sucked into sieve tubes, either by osmosis or through aquaporins, building up the water pressure inside the sieve tube. In the phloem unloading zone, the opposite process takes place. The resulting gradient of water pressure drives the phloem sap flow in sieve tubes along the plant. It is worth noting that the osmotic potential of the phloem sap may depend not only on sucrose concentration but also to level of potassium ions (Grange and Peel 1978; Lang 1983). The mechanism described above assumes a passive role of sieve tubes in long-distance transport. Actually, sieve tubes do participate in the transport albeit indirectly, by reloading the sucrose leaking from the sieve tubes in transport phloem (Minchin et al. 1984; Minchin and Thorpe 1987; van Bel 2003; Ayre 2011).

The above mechanism describes well the long-distance transport of solutes of low molecular weight. However, phloem transport of macromolecules such as proteins and nucleic acids seems to be different from the former, since endogenous macromolecules are often targeted to a given organ or tissue, while the mass flow mechanism assumes an even distribution of phloem sap to sinks depending only on a sink's strength. The destination-selective movement of CmPP16 (*Cucurbita maxima* phloem protein 16) into roots (Aoki et al. 2005) and directed transport of PSTVd (potato spindle tuber viroid) into sepals (Zhu et al. 2002) are both examples of macromolecule long-distance transport which, at first sight, cannot be explained in terms of the mass flow mechanism. The simplest explanation is that unloading of some macromolecules in sink tissues could be strictly controlled (Chen and Kim 2006). It is also possible that sieve tubes are not continuous files of sieve elements but rather a series of sieve tubes divided by zones of unloading and reloading into subsequent sieve tube. These zones could serve as control points to redirect some macromolecules. The idea that sieve tubes could be discontinued at some regions came from modeling. Theoretical considerations demonstrated that sieve tubes longer than several meters could not support the observed flow rates. To overcome the problem, Lang (1979) proposed that long-distance transport goes along a series of shorter sieve tube files. The idea was revitalized recently (Thompson and Holbrook 2003) basing on theoretical considerations. The very existence of discontinuous sieve tubes has not been verified experimentally, but their possible biological importance is unquestionable in the light of destination-selective long-distance transport of macromolecules. One may

hypothesize that zones of phloem unloading/reloading could serve as transshipment stations redirecting macromolecules to their individual destinations.

1.5 Short-Distance Symplasmic Transport in Selected Plant Tissues

The cell-to-cell symplasmic transport is commonly assumed to be strictly local, with any transport at distances exceeding a pair of cells being only possible through sieve tubes. There are, however, many examples of tissues where symplasmic transport covers the distance of several or even more cells without participation of sieve tubes and therefore could justifiably be dubbed short-distance transport and its specific mechanisms. As it was discussed above (see Sect. 1.4.2.3), the diffusive cell-to-cell transport is much less effective than the mass flow in sieve tubes. Therefore, the question arises on the adaptations of some tissues to short-distance transport. Three cases of tissues employing the short-distance transport are presented below shortly: secretory structures, the photosynthate path to the vein in C4 plants, and paraveinal mesophyll. Separate chapters consider symplasmic communication functioning in xylem rays (Chap. 4) as well as phloem loading and unloading (Chap. 5).

1.5.1 External Secretory Structures

Plants secrete diverse substances outside their body. This may simply be a means of dealing with unfavorable external conditions, as when salt is excreted by plants growing on salt marshes, but often is an important aspect of normal physiology, as in examples below. Owing to their toxicity, some secreted substances may be involved in the protection against herbivores (Adler 2000). Other compounds are attractive for pollinators or protecting insects such as ants (Wist and Davis 2006). To secrete these substances, plants have evolved specialized structures—salt glands, salt trichomes, and glandular trichomes. Also staminal hairs may secrete attractive substances apart from other functions they play (Naidoo et al. 2011). All these secretory organs of different structure (Beck 2010) carry out intensive transport, often of symplasmic character. The unique properties of the secretory structures as a model for symplasmic transport studies have been appreciated by many authors (for review, see Waigmann and Zambryski 2000). These structures are easy to handle, including preparation, *in vivo* observations, and microinjection. Hence, a major part of our current knowledge on the topic has come from research on secretory structures.

Salt glands, best known in *Tamarisk aphylla,* are borne on leaves and stems and sunken in the epidermis. They consist of large secretory cells attached to basal collecting cells (Beck 2010). Since the salt glands are covered by a cutinized cell wall, salt has to be transported from the mesophyll to collecting cells symplasmically.

The further transport to the secretory cell is by the same mode (ibid.). Salt secretion to the apoplasm and thus outside the leaf is enabled by secretory cell ingrowth, typical for transfer cells (ibid.). Salt trichomes sit on buds, young green stems, and leaves in many species of *Atriplex* (Smaoui et al. 2011). A salt trichome consists of stalk cells embedded in epidermal cells and a large bladder cell. The latter accumulates salt in the central vacuole and releases its content by cell rupture, not by secretion into the apoplasm as is the case with salt glands (ibid.). The salt accumulation in secretory cells seems to be supported by an active salt-concentration step possibly performed by the tonoplast (Echeverria 2000). Participation of endocytosis and release of the vesicle content to the vacuole has been suggested recently as well (Smaoui et al. 2011). The difference in the salt release mechanism between salt glands and salt trichomes notwithstanding the mode of salt transport into and throughout the secretory structure of both types is symplasmic. This mode of transport is imposed by the highly cutinized cell walls separating the gland from the epidermis and mesophyll (Beck 2010).

Nectary trichomes represent another type of secretory glands. Nectaries are highly variable in morphology, position on the plant body, and the role they play. The transport of pre-nectar to the nectaries may be apoplasmic (eccrinous), like in the floral nectaries of *Digitalis purpurea,* rather large structures of 40–50 cells containing phloem unloaded apoplasmically (Gaffal et al. 2007) or symplasmic (granulocrinous) (ibid.) like in nectary trichomes where symplasmic transport dominates (for review, see Waigmann and Zambryski 2000). In nectary trichomes, several adaptations are observed likely to facilitate efficient symplasmic transport, including a high abundance of plasmodesmata linking the cells, from the basal, through stalk to secretory ones (ibid.). The high plasmodesmal frequency found in leaf trichomes of different types might be related to the formation of secondary plasmodesmata, as found for leaf trichomes in tobacco during the sink/source transition (Roberts et al. 2001). Thus, the high density of plasmodesmata seems to be the most important feature of tissues carrying out intensive symplasmic transport. It is in line with theoretical considerations on the mechanism of control of phloem unloading in sinks (Patrick 1997), which indicate that the number of plasmodesmata is the determinant of efficiency of symplasmic transport.

Another example of secretory structures where symplasmic transport was studied thoroughly is the staminal hair of *Setcreasea purpurea* (Tucker 1982, 1987; Tucker et al. 1989; Tucker and Tucker 1993). Staminal hairs are filamentous files of cells differing in length, shape, and other anatomical traits; they aid pollination by attracting pollinators visually or chemically, by excretion of attractants. Studies on the staminal hairs of *S. purpurea* showed that the main mode of transport of low molecular weight solutes through plasmodesmata is diffusion (Tucker et al. 1989) and cytoplasmic streaming plays no role in cell-to-cell transport (Tucker 1987). The data regarding symplasmic transport obtained by studying staminal hairs should not be viewed as representative for other secretory structures, as they differ from each other in many important details. The diffusion coefficient for movement through plasmodesmata in staminal hairs was found not to depend on the direction (Tucker et al. 1989). However, unidirectional transport from leaf cells to the

trichome and from the basal trichome cell to the distal cell was observed, respectively, in *Abutilon* nectaries (Terry and Robards 1987) and tobacco trichomes (Christensen et al. 2009). This shows that secretory structures may differ in the mechanism of transport and/or its regulation. Another example of a specific symplasmic transport mechanism in secretory structures is the cell-to-cell transport with participation of desmotubules in cotton extrafloral nectary trichomes (Waigmann et al. 1997). This is one of the several examples of "open" desmotubules countering the claim of numerous authors, founded on their observations, that the desmotubules are static, appressed structures not engaged for transport through the plasmodesma (Gunning and Overall 1983; Tilney et al. 1991; Botha et al. 1993; Overall and Blackman 1996; Ding 1998). In that context, one should mention the discovery of Lazzaro and Thomson (1996) on a vacuolar-tubular continuum from base to tip of chickpea trichomes. According to those authors, the vacuolar-tubular continuum could represent the path for solute diffusion through plasmodesmata via a channel in the center of the axial component. In the light of the above observations, it seems likely that the desmotubule could act as a pathway for low molecular weight solutes, at least in some types of secretory structures. Far from being uniform, plasmodesmata exhibit features unique to only a certain type of secretory structures. For instance, the plasmodesmata linking cells of staminal hairs do not transport aromatic amino acids and are even blocked by them (Tucker 1982), while their transport through nectary plasmodesmata is efficient (Terry and Robards 1987). Trichome plasmodesmata in *Nicotiana clevelandii* differ much in ultrastructure (Waigmann et al. 1997) and protein transport ability (Waigmann and Zambryski 1995) from those in the mesophyll. These data show that the only common feature of secretory structures of different types specialized in efficient symplasmic transport is just a high frequency of plasmodesmata, while other may differ.

1.5.2 Photosynthate Path to Vein in C4 Plants

The C4 pathway enhances photosynthetic efficiency, particularly under conditions promoting closing of stomata, i.e., in open, dry, and hot habitats, when both low internal carbon dioxide concentration and high temperature would promote photorespiration in C3 plants (Pearcy and Ehleringer 1984; Sage and Pearcy 2000). This is realized by increasing CO_2 concentration at the site of the dual-activity enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, which tips the balance between ribulose-1,5-bisphosphate carboxylation and oxygenation in favor of the former. The C4 pathway is distributed between two types of cells: Kranz mesophyll (KMS) and chlorenchymatous bundle sheath (BS). The former tissue is the site of primary carbon assimilation (PCA) and the latter of primary carbon reduction (PCR). PCA is the carboxylation of phosphoenolpyruvate catalyzed by phosphoenolpyruvate carboxylase resulting in the formation of C4 organic acids. These acids are then decarboxylated, and the released CO_2 is consumed again by RuBisCO, both reactions in BS cells, thereby introducing carbon to the Calvin cycle (Furbank and

Foyer 1988). The apparently futile cycle of carboxylation-decarboxylation split between KMS and BS cells is the crucial component of the C4 mode as it allows building in BS cells CO_2 concentration severalfold higher than the ambient one. To aid the metabolic "pumping" of CO_2 into BS cells, specialized physical barriers prevent its leakage from the BS. This is achieved by either suberinization of the BS cell walls or maximizing the distance between the KMS and BS chloroplasts (Leegood 2000). There are three main subtypes of C4 photosynthesis: NADP-ME and NAD in dicots and monocots and PEP-CK, only in monocots. Their names derive from the type of enzyme catalyzing malate decarboxylation. For details, the reader should consult other reviews (Furbank and Foyer 1988; Leegood 2000; Sage and Pearcy 2000; Griffiths et al. 2013).

The basic architecture of KMS and BS tissues in relation to each other is rather simple: the two photosynthetic tissues form concentric cylinders—the bundle sheath is located around the veins and is then encircled by a layer of Kranz mesophyll cells (Botha 2005). In some species, an additional layer of mestome sheath can be located between the bundle sheath layer and the vein (ibid.). Beside its role in C4 photosynthesis, such tissue architecture aids efficient exchange of photosynthetic intermediates between KMS and BS cells as well as facilitates the delivery of sucrose to companion cell/sieve element complex. Here, the Haberlandt's principle of expeditious translocation is realized as the transport of synthetic product follows the shortest and most efficient route (after Cittadino 1990).

The transport of C4 intermediates is likely to proceed exclusively through the numerous plasmodesmata crossing the KMS/BS cell interface, since the suberin lamellae often (but not always) decorating the cell walls of BS cells in C4 plants should exclude the apoplasmic mode of transport (Hattersley 1987; Hattersley and Browning 1981, but see Eastman et al. 1988a, b). These plasmodesmata are often simple ones, grouped in pit fields. The KMS/BS plasmodesmata in C4 plants are generally more numerous than their analogues in C3 plants (Botha 1992; Botha and van Bel 1992; Cooke et al. 2000), which positively correlates with the high photosynthesis rate of C4 plants (Botha 1992). Some authors insist that the KMS/BS plasmodesmata are of secondary origin since these two types of cells represent distinct lineages (Dengler and Taylor 2000). Cooke et al. (2000) even argued that the evolution of the C4 type of photosynthesis was only possible in angiosperms because of their unique ability to form secondary plasmodesmata (however, for arguments for secondary plasmodesmata formation in gymnosperms, see above, Sect. 1.3). Others (Botha 2005), however, pointed out that the KMS/BS plasmodesmata are only secondarily modified ones. The frequency of the KMS/BS plasmodesmata in C4 plants shows some adaptability, correlating positively with the demand for more efficient transport through the cell interface: it increases when plants are grown at high light intensities (Sowiński et al. 2007) and at low temperatures (Sowiński et al. 2003). Regardless of the mechanism of formation of KMS/BS plasmodesmata in C4 plants, the high frequency of these plasmodesmata seems to be the determining factor of the high fluxes of photosynthates through the corresponding cell interface and hence the high photosynthetic rate observed in C4 plants.

For decades, simple diffusion was widely accepted as the mode of transport of photosynthetic intermediates through the KMS/BS plasmodesmata. This mechanism was recently questioned on theoretical grounds as not efficient enough for the observed transport fluxes (Sowiński et al. 2008) through the cell interface. Simple calculations using experimentally derived parameters, such as solute concentrations and plasmodesma frequency and dimensions, gave maximal allowable flow rates orders of magnitude below the real ones. Paradoxically, none of the possible alternative transport mechanisms (ibid.), apoplasmic, vesicular, or bulk flow within the vacuome (see Sect. 1.2, above), have been found to be involved in the exchange of photosynthetic intermediates between KMS and BS cells (Szczepanik and Sowiński, unpublished data). This discrepancy indicates that some as yet unknown factors must be in action. One possibility is that the diffusive transport through the KMS/BS interface is facilitated by local accumulation of the photosynthetic intermediates near the pit fields. A possible role of peripheral reticulum concentrated in chloroplast protrusions in directing photosynthates toward plasmodesmata was discussed already by Kursanov (1984). Alternatively, the transport could be powered by superdiffusion involving active cellular processes. A conveyer-belt-like movement of the ER membrane along the plasmodesmata and cytoskeleton elements could enhance the cell-to-cell transport (Roberts 2005). In that context, one should note a recent report of Kramer et al. (2007) who found cell-to-cell movement of low molecular weight solutes much faster than estimated before. Apparently, our understanding of the mechanism of transport through plasmodesmata is far from comprehensive.

The KMS/BS plasmodesmata apparently do not stand out in functional properties, as their SEL is around 850 Da (Weiner et al. 1988), similar to other plasmodesmata. They often have sphincters located at neck regions (Evert et al. 1977; Olesen 1979; Robinson-Beers and Evert 1991; Botha 2005; Sowiński et al. 2007). The sphincters can be located externally or internally of the plasmodesma at either one or both sides. Their role is not clear since relevant experimental data are scarce. A role has been reported for external sphincters in *Betula pubescens* where their strong swelling led to the inactivation of shoot apical meristem by abrogation of cell-to-cell communication (Rinne and van der Schoot 1998). Swelling of internal sphincters at the KMS/BS boundary in cold-treated maize was accompanied by a decrease of photosynthetic rate and less efficient transfer of ^{14}C-photosynthates to the phloem (Bilska and Sowiński 2010). That was is in line with the theoretical considerations of Anisimov and Egorov (2002) who proposed that the degree of neck region opening determines the flow rate of molecules through plasmodesmata. Also, Botha (2005) was of the opinion that internal sphincters were responsible for controlling the flow rates through the KMS/BS cell interface. More experimental data are badly needed to fully uncover the role of sphincters in the regulation of exchange of C4 photosynthetic intermediates between the KMS and BS cells.

The pre-phloem loading transport of sucrose in C4 plants is also symplasmic and comprises the route from the site of its synthesis, KMS, through BS to the vascular parenchyma (VP) cells. The BS/VP plasmodesmata are less numerous than the KMS/BS ones (Botha 1992; Botha and van Bel 1992; Sowiński et al. 2007).

They seem to be of secondary origin as these cells develop from distinct lineages (Dengler and Taylor 2000) and their frequency increases with the growth light intensity (Sowiński et al. 2007). Studies on the SXD1 maize mutant showed that the BS/VP plasmodesmata may play a crucial role in the export of photosynthates from leaves (Russin et al. 1996; Botha et al. 2000). The mutant shows excessive accumulation of sucrose and starch in leaf blades accompanied by reduction of phloem export and has abnormal ultrastructure of BS/VP plasmodesmata (Russin et al. 1996). The observed cessation of symplasmic transport from the bundle sheath cells to the vascular parenchyma was most probably caused by accumulation of callose at the cell interface (Botha et al. 2000). Further studies showed that the *SXD1* gene encodes a protein involved in chloroplast-to-nucleus signaling (Provencher et al. 2001) and is a homolog of VTE1, whose mutation in Arabidopsis and *Synechocystis* (Porfirova et al. 2002; Sattler et al. 2003) results in tocopherol deficiency. An impairment of photosynthate export due to RNAi-induced tocopherol deficiency was also found in potato (Hofius et al. 2004). The *VTE1* mutation in Arabidopsis led to the reduction of photoassimilate export as well, but only at low temperature (Maeda et al. 2006). This is in line with the hypothesis that ROS accumulation (here caused by tocopherol deficiency in the *SXD1* or *VTE1* mutants) could activate signaling pathways leading to the closure of plasmodesmata (Benitez-Alfonso et al. 2011). In the context of C4 photosynthesis regulation, it also shows that photosynthetic processes could control the export of photosynthates by modulating of the properties of symplasmic pathway.

The last stage of pre-phloem transport is the uptake of sucrose into sieve tubes. In dicots, the vein ultrastructure has been classified by Gamalei (1989) as type 2c (low plasmodesmata frequency at the parenchyma/companion cell interface, high plasmodesmata frequency at the mesophyll/bundle sheath interface) evolved from type 2a (low plasmodesmata frequency at the parenchyma/companion cell interface) in response to dry environment. However, most data on phloem loading come from study on dicots, while C4 grasses and in fact all monocots were neglected in considerations on the evolution of the phloem loading mode. In grasses, two forms of sieve tubes can be distinguished in small and intermediate veins, i.e., those involved in phloem loading (Evert et al. 1996): thick-walled sieve tubes and thin-walled sieve tubes (Evert et al. 1978; Botha 1992; Botha and van Bel 1992). The thick-walled sieve tubes adjoin VP cells and are often connected to them by plasmodesmata, which suggests symplasmic contact. However, the functions of the thick-walled sieve tubes are not clear yet; possibly they are not involved in long-distance transport (Fritz et al. 1983) and are less functional than thin-walled tubes (Matsiliza and Botha 2002). The thin-walled sieve tubes adjoin companion cells. The phloem loading of sucrose in C4 plants seems to be apoplasmic, although some reports showed symplasmic continuity between parenchyma and companion cells, at least in C4 grasses (Botha 1992; Sowiński et al. 2001, 2003). This would allow symplasmic phloem loading, provided the plasmodesmata linking vascular parenchyma and companion cells are functional. Direct observations of symplasmic movement of fluorescent dyes to the sieve tube/companion cell complex are lacking in C4 plants. However, in barley, a C3 grass, the companion cell/sieve element complex in the third leaf was

shown not to be symplasmically isolated in some regions (Haupt et al. 2001). We have found recently in *Zea mays* spp. *indentata* (Bilska and Sowiński, unpublished data) that the KMS, BS, and VP cells plasmolyzed at a higher sorbitol concentration than did the companion cell/sieve element complex. Thus, in that maize subspecies, the sucrose transport from mesophyll to vein possibly does not need to go against a concentration gradient, similarly as in some species showing neither apoplasmic nor polymer trapping phloem loading (Turgeon and Medville 1998). The passive mode of symplasmic phloem loading has been shown to be associated with the tree growth form (Davidson et al. 2011). It seems that under conditions favoring photosynthesis in maize leaves, sucrose could be exported along its concentration gradient (Troughton and Currie 1977; Sowiński 1998). Hence, the hypothesis on the participation of symplasmic transport in phloem loading in C4 grasses needs further verification.

The mechanism of transport of C4 photosynthates and the export of sucrose from leaves in C4 plants is of particular importance in the light of recent attempts to introduce the C4 photosynthesis into C3 plants, since both the ultrastructure of the leaf and the use of particular transport mode could be additional obstacles in that effort (for review, see Langdale 2011).

1.5.3 Paraveinal Mesophyll

Paraveinal mesophyll (PVM) is a single-cell layer separating palisade and spongy mesophyll in the plane of minor veins in several legume species. The term PVM was coined by Fischer (1967) and is synonymous with the original *Mittelschicht designation* introduced by Kopff (1892, after Rutten et al. 2003). In recent years (Rutten et al. 2003; Leegood 2008), a related term EBS (extended bundle sheath) is also used which underlies the origin of the tissue. In fact, EBS is not exactly the same, since the former concerns a well-defined cell layer in legume species, while the latter refers to a range of extensions of bundle sheath cells that are widespread in nonlegume species as well (Rutten et al. 2003).

The paraveinal mesophyll is found mostly in *Fabaceae* (Fischer 1967; Brubaker and Lersten 1995; Lansing and Franceschi 2000), but similar tissue has been reported in other taxa as well (Kevekordes et al. 1988; Rutten et al. 2003). The anatomy, ultrastructure, and physiological role of PVM were studied most thoroughly in the soybean (*Glycine max*). PVM comprises large, branched cells forming a sort of a flattened lattice (Franceschi and Giaquinta 1983a, b). During a plant's development, the PVM starts to differentiate before palisade mesophyll does (Liljebjelke and Franceschi 1991). Mature PVM cells are highly vacuolated and contain dense cytoplasm rich in rough ER and dictyosomes but with few small chloroplasts with no starch grains (Franceschi and Giaquinta 1983a). PVM cells are interconnected by numerous simple plasmodesmata grouped in pit fields; they are also connected symplasmically with neighboring bundle sheath, palisade, and spongy mesophyll cells (Franceschi and Giaquinta 1983a, b; Lansing and Franceschi 2000).

Several roles proposed for the PVM have been tested experimentally. One hypothesis suggests that PVM accumulates proteins in vacuoles and is specialized in storage of nitrogenous compounds (Franceschi et al. 1983; Klauer et al. 1991; Klauer and Franceschi 1997; Voo et al. 2013). However, of utmost interest in the context of this monograph is the possibility that PVM is responsible for intermediary transfer of assimilates from the palisade mesophyll to phloem in species with the palisade mesophyll composed of cells forming multiple layers and high numbers of cells per unit area (Lansing and Franceschi 2000). This would be in line with the Haberlandt's principle of expeditious translocation (see Sect. 1.5.1) as PVM is localized between the palisade and spongy mesophyll in close contact to a phloem. In contrast, Fisher (1970) suggested that non-photosynthetic cells such as PVM actually delay the transfer of photosynthates between the photosynthetic pool and transport pool. The Haberlandt's hypothesis was confirmed by $^{14}CO_2$ pulse-chase experiments (Lansing and Franceschi 2000). That the EBS is part of the transport route to phloem was also confirmed in *Ricinus communis* (a nonlegume species) with the use of the iron-chelator nicotianamine as a marker of mass transport (Rutten et al. 2003). The presence of numerous plasmodesmata linking PVM cells with one another and with surrounding tissues speaks in favor of symplasmic transport of photosynthates via PVM to phloem. This concept could gain further support if detailed data were available on the abundance of plasmodesmata in different types of PVM cell interfaces. To the best of my knowledge, no such analysis has been reported. Also, no direct observations have been reported on the symplasmic transport of a fluorescent dye through the PVM tissue in *Glycine max*, the model species for PVM studies. According to Lansing and Franceschi (2000), cytoplasmic streaming observed in the large PVM cells should improve the efficiency of symplasmic transport in the tissue. To sum up, PVM seems likely to act both as a high-capacity symplasmic route for photosynthates and as a protein store at some developmental stage, since these functions need not be mutually exclusive.

1.6 Conclusions

Vascular plants demonstrate the highest level of symplasm integrity including the most efficient transport inside the protoplast syncytium. It seems, however, that no unique mechanism of symplasmic transport has evolved in higher plants, since also nonvascular plants use the same mechanisms of cell-to-cell, short-distance, and even long-distance transport. Despite well over a century of studies, several old and new concepts still await to be clarified, including but not limited to these: desmotubule as a transport pathway (open or closed?), mechanism of transport through plasmodesmata (diffusion, superdiffusion, other?), transport of organelles through plasmodesmata (whether and how big organelles might move through plasmodesmata?), the role of diffusion versus cytoplasmic streaming in short-distance transport, phloem continuum (a continuous sieve tube along the plant body or a file of distinct sieve tubes?), and the ability of lower plants, fungi,

and animals to modify symplasm integrity (are organisms other than higher plants able to eliminate primary plasmodesmata and form secondary plasmodesmata? Do plasmodesmata of organisms other than higher plants contain desmotubules?). Answers to these questions could change our present understanding of symplasmic transport in vascular plants.

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Chapter 2
Developmental Control of Plasmodesmata Frequency, Structure, and Function

Katrin Ehlers and Maike Große Westerloh

Abstract The plant's cell connections called plasmodesmata mediate symplasmic communication processes which play a crucial role in the control of plant development. Several developmental regulators, including a variety of transcription factor proteins and sRNA species, have been shown to move through plasmodesmata in order to regulate gene expression non-cell-autonomously on the transcriptional and posttranscriptional level. The symplasmic exchange of such regulatory molecules is a crucial element in the complex molecular networks controlling plant growth and morphogenesis. It is generally accepted that plasmodesmal communication is essential for the coordination of cell-division activity, cell-fate specification, tissue patterning, and organogenesis. Dynamics of the plasmodesmal networks in the developing tissues are supposed to facilitate modulations of the intercellular communication pathways which correlate with the developmental requirements. The symplasmic organization can be modulated to cause morphogenetic switches in response to environmental or endogenous signals. In the present review, we summarize the distinct modes by which structural and functional alterations of the plasmodesmal networks can be achieved, and we discuss possible molecular control mechanisms. Moreover, we will give an overview on the programmed developmental changes in the number, structure, and in the functional state of plasmodesmata which occur in growing plant tissues, including embryos, leaves, roots, and shoots during primary and secondary growth, as well as during the transition to flowering.

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2.1 Introduction

Evolution of multicellularity was one of the most significant innovations in the history of life. The cells of multicellular animal or plant organisms practice division of labor and developed into manifold highly specialized cell types which are perfectly adapted to their divergent functional tasks (e.g., Ispolatov et al. 2012; Rueffler et al. 2012). Since evolution of multicellularity involves the transition from cellular individuality and autonomous functioning to a coordinated mode of cellular operation, the concomitant development of sophisticated intercellular communication systems was a logical consequence. Intercellular communication guarantees the collaborative functioning of the cells within the multicellular organisms, cooperative responses to exogenous and endogenous stimuli, and controlled developmental events (reviewed, e.g., in Ehlers and Kollmann 2001; Benitez-Alfonso et al. 2011; Burch-Smith et al. 2011a; Burch-Smith and Zambryski 2012; Maule et al. 2012; see also Chaps. 1 and 7).

The plant's cell connections, called plasmodesmata, interconnect the cytoplasm of adjacent cells to a symplasmic continuum. Plasmodesmata are even more sophisticated than the analogous structures in animals, called gap junctions, although both types of cell connections function as communicating junctions. In contrast to gap junctions, plasmodesmata do not only mediate electrical coupling (e.g., van Bel and Ehlers 2005) and the diffusional intercellular exchange of small metabolites but they also govern the targeted or nontargeted symplasmic exchange of macromolecules acting as non-cell-autonomous regulators (Lucas and Lee 2004; Oparka 2004; Ruiz-Medrano et al. 2004; Lucas et al. 2009). Ever since the discovery that the maize KNOX homeobox transcription factor KNOTTED1 behaves like the movement proteins of several plant viruses and moves cell-to-cell through plasmodesmata (Lucas et al. 1995), a variety of plasmodesma-mobile plant molecules have been identified. These include phloem-specific proteins and a number of plant transcription factors which are able to mediate the transport of their own mRNA and to enlarge the plasmodesmal size exclusion limit (SEL) in several cases (reviewed in Lucas and Lee 2004; Oparka 2004; Jackson 2005; Chen and Kim 2006; Lucas et al. 2009; Burch-Smith et al. 2011a; Xu and Jackson 2010; Wu and Gallagher 2011, 2012). Moreover, sRNA species, like miRNAs and siRNAs, have been shown to move through plasmodesmata to control non-cell-autonomous posttranscriptional RNA silencing (e.g., Hyun et al. 2011). Given these outstanding properties of the plant's cell connections, it is reasonable that essential regulatory tasks have been attributed to plasmodesmata, and plasmodesmal communication is supposed to play a central role in the course of plant growth and development.

2.2 The General Role of Plasmodesmata in the Developmental Control of Plants

Plant growth is understood as the interplay between regulated series of initial cell divisions in the self-renewing meristems and specific differentiation events of the cell derivatives in the differentiation zone (Carraro et al. 2006; Moubayidin et al. 2010; Rost 2011; Perilli et al. 2012). Morphogenetic control has been attributed to hormone signaling—in particular auxin, cytokinin, and gibberellin—in conjunction with differential gene expression (Dodsworth 2009; Vernoux et al. 2010; Bitonti and Chiappetta 2011; Cederholm et al. 2012; Durbak et al. 2012; Petricka et al. 2012; Wu and Gallagher 2012). It is generally accepted that plasmodesmata mediate the symplasmic communication processes required for the non-cell-autonomous control of meristem maintenance, organogenesis, and tissue patterning in developing plant organs. Already the classical laser ablation experiments performed by van den Berg et al. (1995) have clearly demonstrated that cell identity in developing *Arabidopsis* roots is acquired by positional signals which are symplasmically forwarded by the more mature cells to the initials. Recently, mutants of GLUCAN SYNTHASE-LIKE 8 (*GSL8*, *CALLOSE SYNTHASE 10*, *CHORUS*; Guseman et al. 2010) and KOBITO1 (a glycosyltransferase-like protein; Kong et al. 2012), whose plasmodesmata have modified functional properties, have been shown to be defective in proper stomata patterning in *Arabidopsis* leaves. This is presumably due to an irregular escape of cell-fate-specifying transcription factors from stomatal lineage cells via altered plasmodesmata. Similarly, gain-of-function mutations in CALLOSE SYNTHASE 3 (*CALS3/GSL12*) cause functional alterations of plasmodesmata and defects in root development (Vatén et al. 2011).

In the past years, complex molecular control networks have been unraveled, which control the stem cell maintenance and the organogenesis at the shoot apical meristem of the model plant *Arabidopsis thaliana* (SAM; e.g., Dodsworth 2009; Guo et al. 2010; Yadav et al. 2011; Choob and Sinyushin 2012; Fig. 2.1). The symplasmic transport of the homeobox transcription factor WUSCHEL (WUS; Yadav et al. 2011) and of KNOTTED1-like homeobox proteins, like SHOOT MERISTEMLESS (STM; Kim et al. 2003) and KNOTTED1-LIKE IN ARABIDOPSIS THALIANA/BREVIPEDICELLUS (KNAT/BP; Kim et al. 2003), plays a central role in this developmental control network.

As shown in Fig. 2.1., the constant cell number in the population of stem cells at the SAM is maintained through the WUS/CLAVATA (CLV) signaling pathway (e.g., Dodsworth 2009). WUS is both necessary and sufficient for stem cell specification. *WUS* is expressed in a few cells of the organizing center/rib zone from where the protein migrates symplasmically (1) in apical direction into the superficial cell layers up to the outermost L1 and (2) in lateral direction into at least two adjacent cell layers towards the peripheral zone to form a protein gradient (Yadav et al. 2011). The WUS signal stimulates the expression of the signal glycopeptide CLV3 in the stem cells through direct transcriptional activation (Yadav et al. 2011). CLV3,

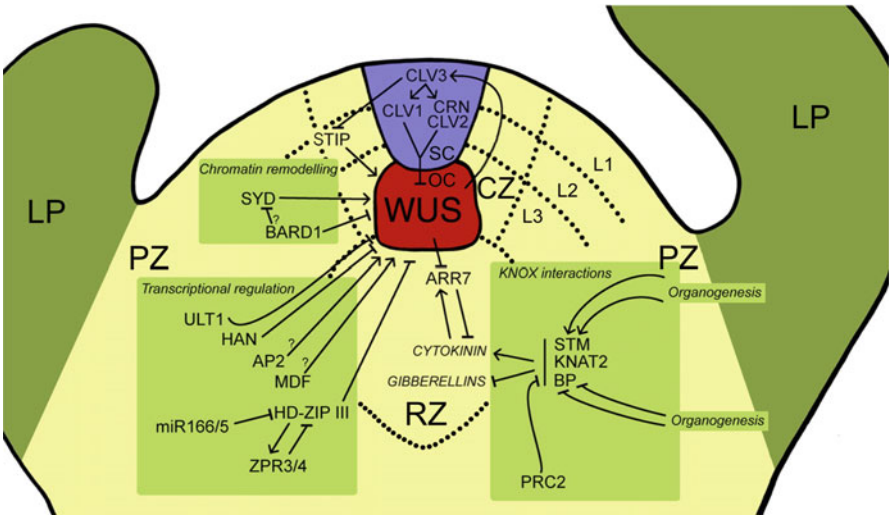

Fig. 2.1 The molecular network of SAM regulation in *Arabidopsis*. CZ: central zone organized in three clonally distinct cell layers (L1–L3), LP: leaf primordia, OC: organizing center, PZ: peripheral zone showing fast cell proliferation and initiation of lateral organs, RZ: rib zone providing the cells for the differentiating stem, SC: stem cells. The SC population at the shoot apex (*blue color*) is maintained through the WUSCHEL (WUS)/CLAVATA (CLV) feedback signaling explained in the text. It includes symplasmic transport of WUS from the site of production (*red*) to adjacent cells (*blue* and *yellow*). *WUS* expression is further regulated through various transcription factors and chromatin remodeling factors. STIMPY (STIP), a WUS-related transcription factor, increases *WUS* expression and is subject to negative regulation by the CLV genes. The floral homeotic protein APETALA 2 (AP2) and the arginin/serin-rich (RS) domain protein MERISTEM DEFECTIVE (MDF) also interact with the WUS/CLV pathway, probably through positive regulation of *WUS*. ULTRAPETALA1 (ULT1), a SAND-domain transcription factor, and HANABA TARANU (HAN), a GATA-3-like transcription factor, suppress *WUS* expression through CLV-independent pathways. Some class III homeodomain-leucine zipper (HD-ZIP III) transcription factors also negatively regulate WUS and are themselves negatively regulated by RNA silencing through miRNA (miR165/166) and by the competitive inhibitors LITTLE ZIPPER proteins (ZPR3/4). Through direct binding to the *WUS* promoter SPLAYED (SYD), a SNF2 class ATPase with chromatin remodeling function, and BRCA1-ASSOCIATED RING DOMAIN 1 (BARD1) regulate *WUS* expression either positively or negatively. BARD1 may also exert its function through suppression of SYD activity. The integrated molecular network of SAM regulation also includes direct links between transcription factors and phytohormone activity. WUS directly represses the cytokinin-induced ARABIDOPSIS RESPONSE REGULATOR 7 (ARR7), which enhances cytokinin activity to stimulate cell division in the SAM. Plasmodesma-mobile KNOTTED1-like homeobox (KNOX) transcription factors in the SAM, like SHOOT MERISTEMLESS (STM) and KNOTTED1-LIKE IN ARABIDOPSIS THALIANA/BREVIPEDICELLUS (KNAT1/BP, KNAT2), stimulate cytokinin biosynthesis to promote cell divisions and suppress gibberellins to inhibit cell differentiation and formation of lateral organ primordia. Expression of the *KNOX* genes is subject to epigenetic silencing via polycomb repressive complex 2 (PRC2) and can be controlled by other transcription factors that regulate organogenesis of leaf primordia and promote leaf identity [modified from Dodsworth (2009), with permission from Elsevier]

which belongs to the CLE (CLAVATA3/EMBRYO SURROUNDING REGION-related) protein family, undergoes posttranslational modification to form a small signaling peptide. This peptide is secreted into the extracellular space and binds to CLV1, a leucine-rich repeat receptor kinase predominantly expressed in cells of the rib zone. Alternatively, peptide binding to related receptor complexes composed of CLV2 and CORYNE/SUPPRESSOR OF LLP1 2 (CRN; Guo et al. 2010) and to RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2; Kinoshita et al. 2010) is also possible. To complete the feedback loop, CLV3 perception by the receptors represses the *WUS* transcription by a mechanism that is not well understood, but may include the downregulation of the activity of the phosphatases POLTERGEIST1 and POLTERGEIST1-LIKE 1 (Guo et al. 2010). Further effectors of the complex control network regulating the *WUS* expression in the SAM are described in Fig. 2.1.

Similar models have also been presented for the *Arabidopsis* root apical meristem (RAM; e.g., Bitonti and Chiappetta 2011; Cederholm et al. 2012; Perilli et al. 2012; Petricka et al. 2012), as well as for the floral meristem (e.g., Krizek and Fletcher 2005; Vernoux et al. 2010; Smaczniak et al. 2012; Torti et al. 2012; Wu and Gallagher 2012) and for the lateral cambium meristem (Hou et al. 2006; Nilsson et al. 2008; Elo et al. 2009; Du and Groover 2010; Risopatron et al. 2010; Yordanov et al. 2010; Agusti et al. 2011; Robischon et al. 2011; Suer et al. 2011; Nieminen et al. 2012; Ursache et al. 2013).

That the symplasmic exchange of plasmodesma-mobile developmental regulators is not restricted to transcription factors, but also includes sRNA species, has elegantly been demonstrated for the vascular cell patterning in *Arabidopsis* root meristems, which determines the developmental fate of the meta- and protoxylem, the pericycle, and the endodermis cells (reviewed in Furuta et al. 2012; Fig. 2.2). Here, the GRAS family transcription factor SHORT ROOT (SHR), which is expressed in the developing stele, moves symplasmically in centrifugal direction towards the developing ground tissue of the cortex, where the SCARECROW (SCR) transcription factor is expressed. After the contact of the two proteins in the innermost cell layer of the cortex, a SHR–SCR complex is formed which determines the position of the future endodermis through induction of endodermis-specific gene expression (see Furuta et al. 2012). The complex also activates the transcription of miR165/166 genes, to induce miRNA-mediated RNA silencing of class III homeodomain-leucine zipper (HD-ZIP III) transcription factors, like, e.g., PHABLOSA (PHB), PHAVOLUTA (PHV), REVOLUTA/INTERFASCICULAR FIBERLESS 1 (REV/IFL1), and CORONA (CNA)/ATHB15 (see Furuta et al. 2012), which are well-known developmental regulators. The function of miR165/166 is, however, not restricted to the endodermis cell layer, but the miRNA moves cell-to-cell through plasmodesmata in centripetal direction towards the stele to form a gradient which exerts posttranscriptional control of the HD-ZIP III target proteins in a dose-dependent manner (Carlsbecker et al. 2010; Vatén et al. 2011). The HD-ZIPIII proteins in turn control xylem identity, such that high protein concentrations (low miRNA levels) induce metaxylem identity, whereas low protein

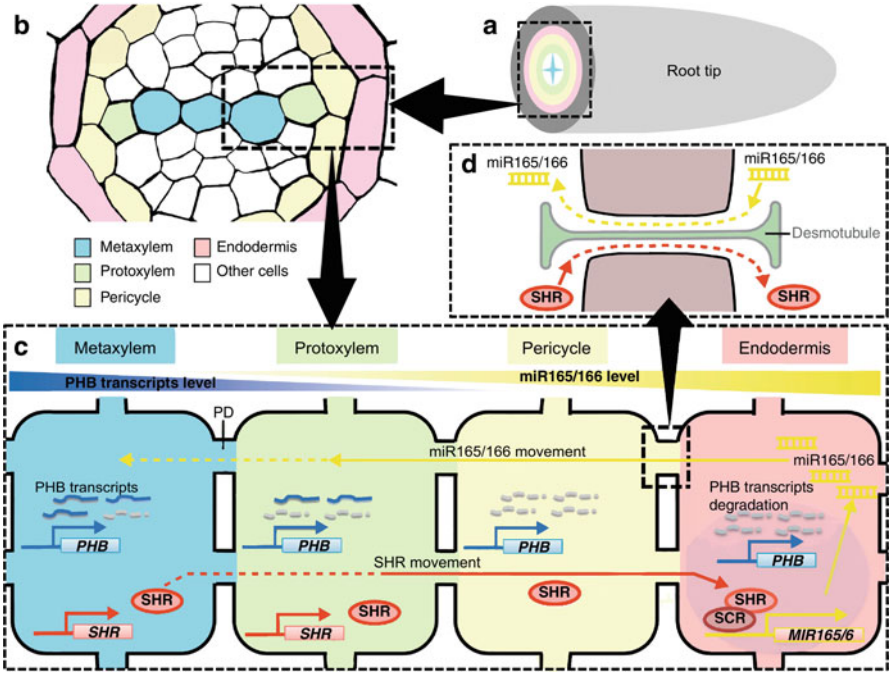

Fig. 2.2 Bidirectional symplasmic transport determines xylem-cell patterning in the *Arabidopsis* root. SHR: GRAS transcription factor SHORT ROOT, SCR: GRAS transcription factor SCARECROW, PHB: HD-ZIPIII transcription factor PHABLOSA, miR165/6: microRNA 165/166. Schematic drawing of the meristematic region of the *Arabidopsis* root tip (**a**) and a detail showing the position of metaxylem, protoxylem, pericycle, and endodermis cells in transverse section (**b**). A molecular model of the tissue patterning is shown in (**c**). SHR is expressed in the stele and moves through plasmodesmata (PD) in centrifugal direction to the future pericycle and endodermis cells. In the endodermis layer, SHR binds to SCR and the complex activates *MIR165/6* expression. The gene product miR165/166 suppresses the level of PHB transcripts in a dose-dependent manner, and it moves symplasmically in centripetal direction to the future pericycle and xylem cells to establish a gradient of PHB activity (Carlsbecker et al. 2010; Vatén et al. 2011). High, medium, and low PHB levels control the fate of metaxylem, protoxylem, and pericycle cells, respectively. A schematic detail of a plasmodesma in a tangential root cell wall is shown in (**d**). Note that the postulated symplasmic transport of SHR and miR165/166 is directed in opposite directions [modified from Furuta et al. (2012), with permission from Elsevier]

concentrations (high miRNA levels) promote protoxylem identity (Carlsbecker et al. 2010). Minimal HD-ZIPIII protein concentrations in those cells which directly adjoin the developing endodermis layer determine the pericycle cell fate.

In this molecular model, the concentration gradients of the two key players, SHR and miR165/166, are established by bidirectional transport processes through plasmodesmata (Fig. 2.2d). Although the symplasmic net flow of the two molecules is

directed in opposite directions, their transport may simply follow the respective diffusional gradients. Yet, targeted transport through plasmodesmata may also occur (e.g., Crawford and Zambryski 2001; Burch-Smith et al. 2011a) and asymmetric plasmodesmal transport capacities have recently been reported for leaf trichomes of tobacco. Here, the small fluorescent dye Lucifer Yellow (457 Da) moves unidirectional in apical direction from the cytosol of the epidermal cell into the basal cell of the trichome, but not vice versa (Christensen et al. 2009). Similarly, transport of photoactivatable GREEN FLUORESCENT PROTEIN (GFP) was found to be polar in apical direction between the basal and apical cell of young tobacco embryos (Li et al. 2013).

To fulfill the regulatory function in the course of developmental processes properly, symplasmic communication through plasmodesmata needs to be strictly controlled. Plasmodesmata do not represent open gates which mediate unhindered symplasmic exchange between the cells. The plant body is rather supposed to function as a complex system of separate symplasmic modules, called symplasmic fields or symplasmic domains (reviewed in Ehlers and van Bel 1999; Ehlers and Kollmann 2001; Pfluger and Zambryski 2001; Kobayashi et al. 2005; Burch-Smith et al. 2011a). At particular cell interfaces of the developing tissues, symplasmic barriers are established by temporary or permanent downregulation of symplasmic transport and by interruption of plasmodesmal connectivity. Individual cells or groups of cells become symplasmically uncoupled from the neighboring subsets of cells to restrict the transport of developmental determinants to the actual target cell domain. Symplasmic isolation has been shown to be crucial for the patterning, the cell-fate determination, and the proper development of stomata guard cells (Willmer and Sexton 1979; Wille and Lucas 1984; Palevitz and Hepler 1985; Guseman et al. 2010; Kong et al. 2012), root hairs (Duckett et al. 1994), and sieve element/companion cell complexes in the transport phloem (van Bel and van Rijen 1994). Duration of the transient symplasmic isolation that occurs in the rapid phase of cotton-fiber elongation has been shown to determine fiber length (Ruan et al. 2004). Moreover, a strict correlation between the plasmodesmal connectivity and the coordination of mitotic activity has been shown for microcalluses of *Solanum nigrum* (Ehlers and Kollmann 2000). Here, symplasmically coupled cells were found to divide synchronously, while asynchronous divisions required the transient uncoupling via plasmodesmata closure. In the dormant cambium of *Populus nigra*, cease of cell-division activity coincides with a drastic reduction of plasmodesmal frequencies, while particularly high numbers of plasmodesmata occur in the tangential walls of the dormant initial layer which will undergo the first synchronous cell divisions in spring (Fuchs et al. 2010a, see below for details; cf. also Fig. 2.6 below). The limited growth of *Azolla* roots (Gunning 1978) and the determinate growth pattern of *Arabidopsis* roots (Zhu and Rost 2000; Rost 2011) have also been ascribed to decreasing numbers of plasmodesmata at the interfaces of the initial cells leading to an increasing symplasmic isolation which restricts the mitotic activity.

A growing body of evidence indicates that programmed changes in the number, structure, and in the functional characteristics of plasmodesmata occur in the

growing plant tissues, including embryos, leaves, roots, and shoots during primary and secondary growth, as well as during the transition to flowering (reviewed, e.g., in Ehlers and Kollmann 2001; Burch-Smith et al. 2011a). The dynamics of the developing plasmodesmata networks facilitate modulations of the intercellular communication pathways which correlate with the developmental coordination of cell divisions and cell differentiation processes and can be modulated to cause morphogenetic switches in response to environmental or endogenous signals (reviewed in Ehlers and Kollmann 2001; Burch-Smith et al. 2011a; Burch-Smith and Zambryski 2012).

2.3 Plasmodesmata Substructure and Pathways for Plasmodesmal Transport

All plasmodesmata are cylindrical, plasma-membrane-lined cytosolic strands of about 30–50 nm in diameter traversing the plant cell walls (reviewed in Overall 1999; Ehlers and Kollmann 2001; Roberts 2005; Fig. 2.3a). While the median part of the cell connections is usually expanded, the plasmodesmal orifices are often constricted to form neck regions which presumably represent the bottlenecks for symplasmic transport. Often, plasmodesmata are surrounded by special cell-wall collars, whose structure and composition differ from the normal cellulose wall (e.g., Badelt et al. 1994; Roy et al. 1997; Orfila and Knox 2000; Roberts 2005; see Fig. 2.4q–s). As a central component, each plasmodesma contains a narrow, tightly curved strand of the endoplasmic reticulum (ER) which is called the desmotubule and has a diameter of only 10–15 nm (Ding et al. 1992a; Tilsner et al. 2011; Fig. 2.3a). It has recently been discussed by Tilsner et al. (2011) whether the extreme, energetically unfavored membrane curvature of the desmotubule is maintained by the association of the desmotubule with plasmodesma-localized components of the cytoskeleton and/or by ER-shaping proteins, like plant homologs of reticulons (RTNs, Sparkes et al. 2010) and DP1/REEP5/Yop1 family proteins (Hu et al. 2008; see also the discussion below).

The space of the cytoplasmic sleeve between the desmotubule and the plasma membrane is restricted by diverse proteins which are associated peripherally or integrally with both membranes (e.g., Faulkner and Maule 2011; Fig. 2.3a). Helically arranged proteins attached to the desmotubule and proteins attached to the plasma membrane are interconnected by spoke-like proteins (Fig. 2.3a). The remaining cytosolic microchannels of about 2.5 nm (Ding et al. 1992a; Overall 1999) are regarded as the main pathways for symplasmic communication between the cells. However, there is also evidence that both, the membrane and the luminal space of the desmotubule, provide alternative pathways for cell-to-cell transport, at least at particular cell interfaces (Grabski et al. 1993; Lazzaro and Thomson 1996; Cantrill et al. 1999; Martens et al. 2006; Guenoune-Gelbart et al. 2008; Barton et al. 2011).

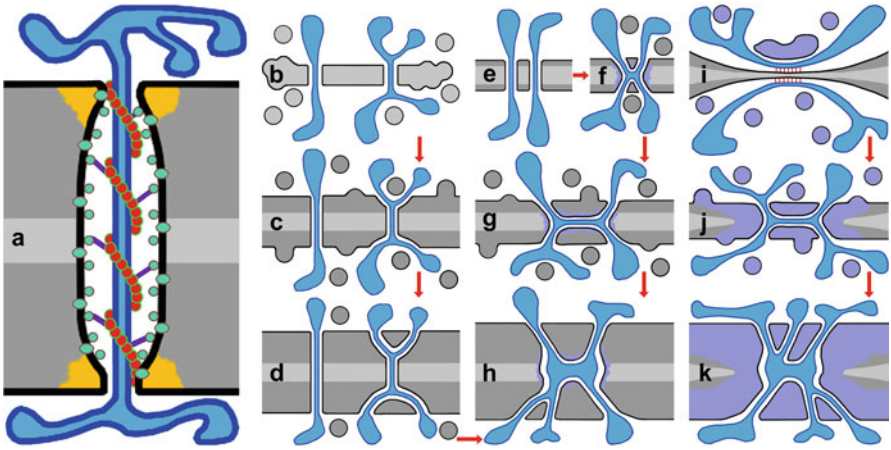

Fig. 2.3 Schematic drawings depicting the general plasmodesmal ultrastructure (**a**) and different mechanisms of the formation of branched plasmodesmal morphotypes (**b**–**k**). The same color code is used in all drawings. *Black*: plasma membrane, *blue*: endoplasmic reticulum (ER)/desmotubule, *dark gray*: primary and secondary cell-wall layers, *light gray*: middle lamellae, *violet*: modified and reconstructed wall material, *white*: cytoplasm. (**a**) A plasmodesma with neck constrictions at the orifices. Callose deposits (*yellow*) are often found in the cell-wall collars around the necks. Proteins attached to the plasma membrane (*green*) and the desmotubule (*red*) are interconnected by spoke-like proteins (*purple*). The cytoplasmic sleeve is restricted to small microchannels. (**b**–**d**) Primary plasmodesmata formed during cytokinesis in the growing cell plate usually have a simple, straight morphology (**b**). Branched morphotypes may develop while the primary plasmodesmata are elongated during thickening growth of the wall, from the incorporation of branched ER cisternae into the new wall layers (**b**–**d**). Median cavities may develop from the lateral dilation of the median plasmodesmal part in the course of wall-expansion growth, giving rise to complex branched plasmodesmal morphotypes (**d**, **h**). (**e**–**h**) Fusion of neighboring simple plasmodesmata during cell-wall expansion (**e**, **f**) is an alternative way to form branched plasmodesmal morphotypes. Initially, they are X-shaped (**f**) or H-shaped (**g**), but they may develop into complex branched plasmodesmata with median cavities and multiple branching planes (**h**). (**i**–**k**) De novo secondary plasmodesmata develop at sites where preexisting cell walls have been thinned and ER cisternae have been attached to the plasma membranes (**i**). After membrane fusion, plasmodesmata formation is driven by the cell-wall (re)construction (**j**, **k**). Note, that plasmodesmal fission is presented in Fig. 2.4l as an alternative mechanism of plasmodesmata formation [Figs. 2.3(**b**–**k**) modified from Ehlers and Kollmann (2001), with kind permission from Springer Science + Business Media]

In contrast, the plasma membrane within the plasmodesmal pore seems to act as a barrier for lateral diffusion of molecules and does not mediate the exchange of fluorescently labeled lipids (Grabski et al. 1993; see Tilsner et al. 2011). This peculiarity might be explained by the enrichment of remorin-containing rafts in the plasmodesmal plasma membrane (Raffaele et al. 2009; Mongrand et al. 2010) and/or by the anchorage of plasma-membrane proteins to cytoskeletal components of

the plasmodesmata, which establishes diffusional barriers according to the "picket fence" model (see Tilsner et al. 2011). Similar to this mechanism known as "cytoskeleton corralling," the limitation of the lateral mobility of plasma-membrane proteins may also be achieved by the interaction with the cell-wall components at the extracellular face, as has recently been shown by Martinière et al. (2012).

In the past years, enormous progress has been made in the identification of the molecular plasmodesmal components which also provide insights into the mechanisms regulating the functional properties of the cell connections (reviewed in Roberts 2005; Burch-Smith et al. 2011b; Faulkner and Maule 2011; Fernandez-Calvino et al. 2011; Tilsner et al. 2011; Burch-Smith and Zambryski 2012). Actin, myosin VIII, centrin, calreticulin, the actin-binding proteins Arp3, tropomyosin, and NET1A, as well as Ca^{2+}-dependent protein kinases have been localized at plasmodesmata and may cause alterations of the size of the plasmodesmal microchannels, possibly in a Ca^{2+}-dependent manner (reviewed, e.g., in Holdaway-Clarke 2005; Faulkner and Maule 2011; Radford and White 2011; cf. also Deeks et al. 2012). Moreover, deposits of the β-1,3-glucan callose can be formed in the cell-wall collars around plasmodesmata (Fig. 2.3a) to constrict their orifices and to reduce the SEL or inhibit communication temporarily (e.g., Roberts 2005; Benitez-Alfonso et al. 2011; Zavaliev et al. 2011; Burch-Smith and Zambrysky 2012; see Chap. 3). In addition to the above-mentioned callose synthases GSL8 (Guseman et al. 2010) and CALS3/GSL12 (Vatén et al. 2011), several plasmodesmal proteins are involved in callose metabolism, such as the callose degrading β-1,3-glucanase (AtBG_ppap, Levy et al. 2007) and the callose-binding PDCB1 (Simpson et al. 2009), both of which are predicted to be anchored to the plasmodesmata membrane by GPI linkers. Remarkably, overexpression of the plasmodesmata-associated class 1 reversibly glycosylated polypeptide (AtRGP2) leads to increased callose deposition at plasmodesmata and to restricted local spread of tobacco mosaic virus via plasmodesmata (Zavaliev et al. 2010). As expected, silencing of *AtRGP2* induces accelerated symplasmic virus spread (Burch-Smith et al. 2012).

Furthermore, spatially defined and/or tissue-specific expression patterns of plasmodesmal proteins may cause different functional plasmodesmal properties correlating with the occurrence of symplasmic domains, as has recently been suggested for two members of the family of PLASMODESMATA-LOCATED PROTEIN1 (PDLP1, *At2g33330* and *Atg04520*) in the *Arabidopsis* shoot apical meristem (Bayer et al. 2008). Conceivably, there are also developmental changes in the plasmodesmal protein equipment which adapt the cell connections to their divergent functional tasks. Differences in the composition of receptor-like proteins (RLPs) and receptor-like kinases (RLKs) located at plasmodesmata (Lee et al. 2005; Amari et al. 2010; Burch-Smith et al. 2011b; Faulkner and Maule 2011; Fernandez-Calvino et al. 2011; Jo et al. 2011; Burch-Smith and Zambryski 2012) may exert control on the cell- and tissue-specific perception and processing of developmental effectors.

2.4 Plasmodesmal Morphotypes and the Mechanisms of Plasmodesmata Formation, Modification, and Deletion

2.4.1 Primary Plasmodesmata

While plasmodesmata have almost identical dimensions and a generally identical architecture, the plasmodesmal morphology is manifold. Simple and straight plasmodesmata can be found beside different types of branched cell connections, and the mechanisms leading to the development of the distinct plasmodesmal morphotypes have often been discussed (for reviews, see Kollmann and Glockmann 1999; Ehlers and Kollmann 2001; Roberts 2005; Burch-Smith et al. 2011a; Figs. 2.3b–k and 2.4l). Primary plasmodesmata formed in the course of cytokinesis mostly have a simple, straight morphology. They develop during cell-plate assembly from the entrapment of ER tubules crossing the plane of the fusing Golgi vesicles (Hepler 1982; Staehelin and Hepler 1996; Ehlers and Kollmann 2001; Roberts 2005; Fig. 2.3b). In the newly generated division walls of different meristematic plant tissues, substructural details were found to be obscured in the most recently formed primary plasmodesmata which often exhibit a vague internal structure (Ehlers and van Bel 2010; Fuchs et al. 2010a, 2011; Fig. 2.4b–k). Dilated median parts, in which discrete internal substructures can be seen, and constricted neck regions may develop later during maturation of the cell connections (Ding et al. 1992a; Roberts 2005), and these structural changes may be accompanied by a downregulation of the plasmodesmal SEL (Ehlers and van Bel 2010; Fuchs et al. 2010a, 2011).

Simple primary plasmodesmata are elongated during thickening growth of the division walls (Ehlers and Kollmann 1996, 2001; Fig. 2.3c, d). As the new wall-matrix material is delivered by Golgi vesicles, straight or branched cytoplasmic ER cisternae connected to the desmotubules are entrapped, and they are incorporated into the cytosolic strands elongating the linear median part of each primary plasmodesma. The shape of the incorporated ER tubules defines the shape of the new plasmodesmal parts in the younger wall layers. Elongated primary plasmodesmata may still have a simple morphology or they show branching planes in the young wall layers (Fig. 2.3d), either on one or on both sides of the cell wall. Multiple branching planes may also occur, but the median cell-wall layers are still traversed by the oldest, straight part of the primary plasmodesma (Ehlers and Kollmann 1996, 2001; Fig. 2.3d). Excessive cell-expansion growth, however, may cause mechanical wall stress, tissue tension, and the loosening of the oldest wall layers (Schopfer 2006) which in turn leads to the lateral dilation of the median part of the branched primary plasmodesma to form an enlarged central cavity in the oldest wall layers (Kollmann and Glockmann 1999; van der Schoot and Rinne 1999a; Ehlers and Kollmann 2001; see Fig. 2.3d, h).

Fusion of laterally adjacent simple cell connections has often been discussed as an alternative way to produce branched plasmodesmal morphotypes (Glockmann and Kollmann 1996; Volk et al. 1996; Itaya et al. 1998; Kollmann and Glockmann 1999; Oparka et al. 1999; Roberts et al. 2001; Ehlers and Kollmann 2001; Roberts 2005;

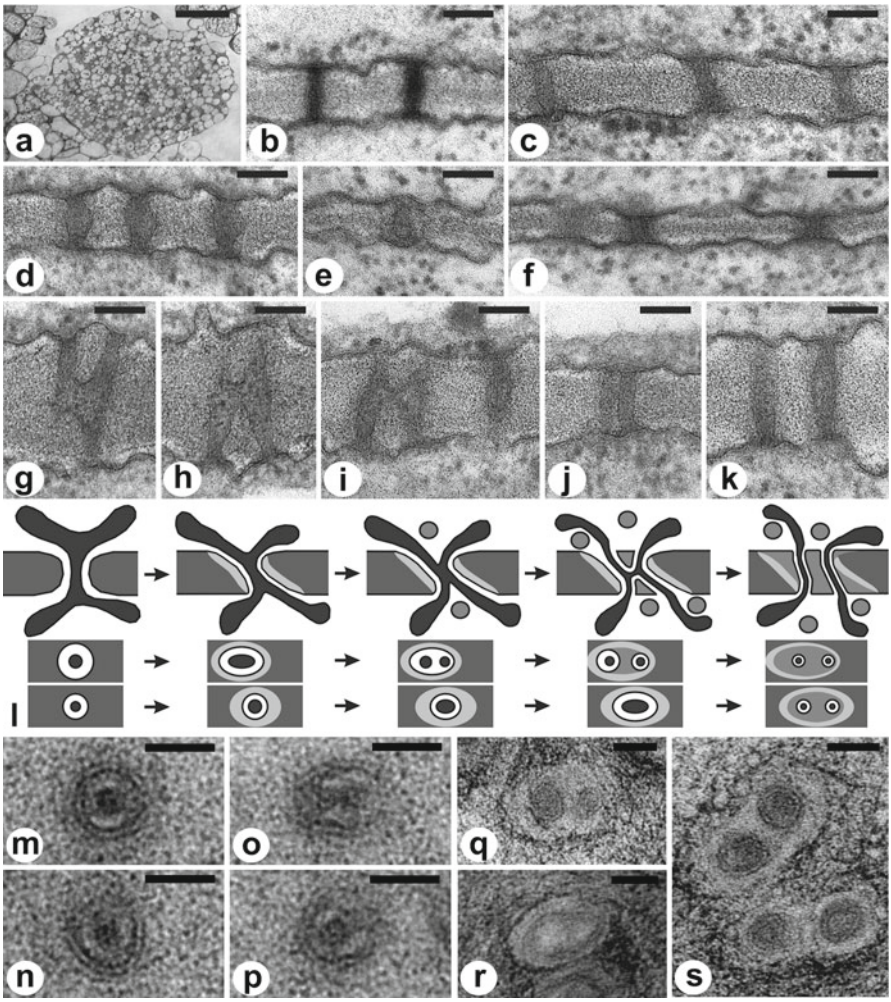

Fig. 2.4 Longitudinal fission of plasmodesmata increases plasmodesmal numbers in developing plant tissues, and intermediate stages of the fission process can frequently be found. (**a**–**k**) Electron micrographs taken from young somatic embryos which were grown on embryogenic calluses of *Molinia caerulea*. The material was cultured and chemically fixed as described by Ehlers et al. (1999). An overview of a young, globular somatic embryo is shown in (**a**). At the cell interfaces of the somatic embryos, straight, perpendicular (**b**) or oblique (**c**) simple cell connections were found besides conspicuously swollen plasmodesmal structures (**d**–**f**). X-shaped and H-shaped plasmodesmal morphotypes with large (**g**, **h**; detected in the same cell wall) or narrow joints (**i**) in the median plane of the wall occurred along with closely adjoining simple cell connections (**j**). These plasmodesmal morphotypes likely represent transitional stages in the process of longitudinal plasmodesmal fission which leads to the formation of laterally adjacent twinned plasmodesmata (**k**). The sequence, in which the images are arranged, was deduced from the chronology of the plasmodesmal development observed in the tomato cambial zone (see Ehlers and van Bel 2010). Plasmodesmata within the same embryonic cell wall showed striking structural similarity to each other, indicating a largely synchronous development of the cell connections. Internal substructures could hardly be seen within these plasmodesmata, which seems to be a typical feature of many

Fig. 2.3e–h). Possibly facilitated by the loosening of the oldest wall layers at the onset of cell expansion, the median parts of the neighboring primary cell connections become slightly dilated and fuse to give rise to an X-shaped or H-shaped plasmodesma (Fig. 2.3e, f). By continuing expansion growth and subsequent wall thickening, more complex branched plasmodesmal morphotypes with enlarged central cavities and multiple branching planes may develop (Fig. 2.3g, h).

2.4.2 Plasmodesmal Fission

The occurrence of X-shaped and H-shaped plasmodesmata need not always be indicative of plasmodesmal fusion processes (Fig. 2.3e, f), and the sequence of developmental events might have been misinterpreted in some cases. As shown in Fig. 2.4l, the branched plasmodesmal morphotypes (Fig. 2.4f–i) may also represent intermediate stages in the process of longitudinal plasmodesmal fission giving rise to closely adjacent pairs of plasmodesmal replicates, called twinned plasmodesmata (Ehlers et al. 2004; Faulkner et al. 2008; Ehlers and van Bel 2010; Burch-Smith et al. 2011a; Fig. 2.4j, k, s). This reverse interpretation of the data is strongly

Fig. 2.4 (continued) young cell connections. (**l**) Schematic drawing showing the model of plasmodesmal replication by longitudinal fission of a simple plasmodesma postulated by Ehlers et al. (2004), and Ehlers and van Bel (2010). The sequence of events as viewed in longitudinal sections is depicted in the *upper row* of drawings. The *median* and *lower rows* of drawings show cross-sectional views of the orifice plane and of the median plane of the plasmodesmata, respectively. Wall-extension growth causes mechanical stress and tissue tension (Schopfer 2006) and induces the loosening of the cell-wall sleeve surrounding the plasmodesma which leads to the deformation of the plasmodesmal strand. As new matrix material is delivered by Golgi vesicles to the growing wall, branched ER cisternae connected to a desmotubule are incorporated and develop into additional plasmodesmal branches. This gives rise to an H-shaped plasmodesma, in which the small median bridge connecting the parallel new branches represents the original plasmodesmal part. Further intense wall extension causes the rupture of the median bridge of the H-shaped plasmodesma, so that a pair of neighboring simple plasmodesmata is formed. In the course of less intense wall-extension growth, the H-shaped plasmodesmal intermediates may also develop into complex branched morphotypes (see Fig. 2.3f–h). (**m**–**s**) Cross-sectional views of plasmodesmata found in the tangential walls of developing ray cells in the cambial zone of *Populus nigra* stems. The observed structures resemble the cross-sectional views predicted in our model for the intermediate stages of plasmodesmal fission (cf. **l**). In normal plasmodesmal cross sections, the plasma membrane, the constricted desmotubule, and particles in the cytoplasmic sleeve can be detected, but no conspicuous cell-wall sleeves were observed in the vicinity of the cell connections (**m**, **n**). Sometimes, however, two desmotubule-structures can be seen within the same, enlarged plasmodesmal pore (**o**), or two narrow paired plasmodesmal strands were found within the same cell-wall sleeve, which seems to consist of loosely arranged material (**q**, **s**). With the upper plasmodesmal pair shown in (**s**), the density of the wall material located between the two plasmodesmal strands differs from the rest of the cell-wall sleeve. Moreover, cross-sectional views with slightly or clearly enlarged desmotubules were observed (**p**, **r**), and the latter are also surrounded by loosened cell-wall-collars (**r**). Scale bars: 50 µm in (**a**); 100 nm in (**b**–**k**); 50 nm in (**m**–**s**) [Fig. 2.4l reprinted from Ehlers and van Bel (2010), with kind permission from Springer Science + Business Media]

supported by recent quantitative studies on the development of plasmodesmata in the cambial zone, where the occurrence of branched and twinned cell connections is restricted to particular cell interfaces and is accompanied by a concomitant doubling of the respective plasmodesmal numbers (Ehlers et al. 2004; Ehlers and van Bel 2010; Fuchs et al. 2010a, 2011; Fig. 2.6). Moreover, as the plasmodesmal numbers increase in the basal walls of leaf trichomes of tobacco, the plasmodesmal arrangement shifts from randomly distributed simple plasmodesmata in the early postcytokinetic wall to pit fields with densely arranged multiply twinned plasmodesmata (Faulkner et al. 2008). These findings point to a (simultaneous) division of existing plasmodesmata rather than to a plasmodesmal fusion. Shifts to pit field aggregates have been reported earlier for the interfaces of growing roots and developing pits of fiber tracheids (Seagull 1983; Barnett 1987).

It remains a matter of debate, whether plasmodesmal fission is achieved by the "active" insertion of new ER strands in immediate vicinity of the existing template plasmodesma pore (Faulkner et al. 2008) or by the "passive" enclosure of desmotubule-linked ER strands among the cell-wall appositions delivered to the expanding cell wall (Ehlers et al. 2004; Ehlers and van Bel 2010; Fuchs et al. 2010a, 2011; Fig. 2.4l). Predicted intermediate stages of an "active" ER insertion, i.e., new ER strands originating from the cytoplasm and ending blindly in the plasmodesmal wall collars or in the plasmodesmal pores (see Fig. 7 in Faulkner et al. 2008), have never been found in extensive electron-microscopic studies on plant embryos and sink leave tissues (Burch-Smith et al. 2011a). This observation supports the alternative "passive" model of plasmodesmal fission, which has striking mechanistic similarities to the models of primary plasmodesmata formation and modification described above (Ehlers et al. 2004; Ehlers and van Bel 2010; compare Figs. 2.3b–h, 2.4l). Supposedly, there is a common principle underlying the developmental alterations of the plasmodesmal number and structure, and the incorporation of ER tubules within growing cell walls actually plays a general role (Ehlers and Kollmann 2001; Ehlers and van Bel 2010). Thus, it would simply depend on the degree of cell-wall expansion, whether (1) the process of plasmodesmal fission is completed (Fig. 2.4l) and pit fields develop from randomly arranged primary plasmodesmata by multiple replications (e.g., Seagull 1983; Barnett 1987; Faulkner et al. 2008) or whether (2) the fission process is arrested in an intermediate stage and is followed by the formation of complex branched plasmodesmata from the X-shaped and H-shaped plasmodesmal intermediates during subsequent wall thickening (Ehlers and van Bel 2010; Fuchs et al. 2010a, 2011; Figs. 2.4l and 2.3f-h).

2.4.3 Secondary Plasmodesmata

Secondary plasmodesmata are defined as those cell connections which originate post-cytokinetically in already existing cell walls (Kollmann and Glockmann 1999; Ehlers and Kollmann 2001; Roberts 2005; Burch-Smith et al. 2011a). Although simple secondary plasmodesmata have also been observed, a complex branched morphology—with a dilated main branching plane in the median wall layer and

additional branching planes in the younger wall layers—has often been considered as a typical feature of secondary cell connections (Monzer 1991; Kollmann and Glockmann 1991, 1999; Ehlers and Kollmann 2001; Roberts 2005; Fig. 2.3k). Some authors generalized this observation and called every complex branched cell connection a secondary plasmodesma, irrespective of its origin (e.g., Ding et al. 1992b; Volk et al. 1996; Itaya et al. 1998). This caused some confusion in the plasmodesmal nomenclature (reviewed in Ehlers and Kollmann 2001; Burch-Smith et al. 2011a), but, meanwhile, there is general agreement that complex branched plasmodesmal morphotypes may develop from both primary and secondary cell connections (cf. Fig. 2.3b–k). Furthermore, it has become a nomenclatory convention to avoid the terms "primary" and "secondary" plasmodesmata whenever the plasmodesmal origin has not been confirmed unambiguously and to classify the observed cell connections merely according to their morphology as "simple," "branched," or "complex branched" as suggested by Ehlers and Kollmann (2001; see, e.g., Roberts et al. 2001; Burch-Smith and Zambryski 2010).

Additional cell connections arising from the lateral division of simple (primary) plasmodesmata (Ehlers et al. 2004; Faulkner et al. 2008; Ehlers and van Bel 2010; Fig. 2.4l) might be regarded as secondary in origin (Burch-Smith et al. 2011a). In most cases, however, it would be impossible to distinguish the newly formed secondary plasmodesmal twin from the original "template" plasmodesma (Faulkner et al. 2008), and the "passive" fission model would even predict that each of the replicated cell connections is composed of both original and newly formed halves (Ehlers et al. 2004; Ehlers and van Bel 2010; Fig. 2.4l). This should be borne in mind when using the term "twinned secondary plasmodesmata" which was suggested by Burch-Smith et al. (2011a) to distinguish the replicated twinned plasmodesmata from "de novo secondary" plasmodesmata developing in cell walls without preexisting plasmodesmal templates.

The mechanism of de novo secondary plasmodesmata formation (Fig. 2.3i–k) has been unraveled by studying fusion walls between heterotypic cells at graft unions (Kollmann and Glockmann 1991) and in protoplast-derived regenerating cell cultures (Monzer 1991). The initial local thinning of the fusion wall removes the physical hurdle between the adjacent cells and enables the close contact of their plasma membranes, which are attached to ER tubules on the cytoplasmic sides (Fig. 2.3i). Fusion of both plasma membranes and ER membranes and the subsequent reconstruction of the degraded wall parts lead to the formation of a secondary plasmodesma with a complex branched structure (Fig. 2.3j, k). Following the same principle as the formation and modification of primary cell connections (Kollmann and Glockmann 1999; Ehlers and Kollmann 2001), cytoplasmic ER tubules connected to the membrane fusion site are incorporated into the newly formed plasmodesmal strands, as the new wall-matrix material is delivered by Golgi vesicles (Fig. 2.3j, k). A similar mechanism has also been postulated for the development of heterotypic secondary plasmodesmata between the *Cytisus purpureus* epidermis and the underlying *Laburnum anagyroides* cells of the monekto-periklinal chimera *Laburnocytisus adamii* (Steinberg and Kollmann 1994) and at the contact interfaces of parasitic flowering plants and their hosts (reviewed in Kollmann and Glockmann 1999; Ehlers and Kollmann 2001). Interestingly, mismatching half plasmodesmata

extending across the wall half of only one cell partner were regularly observed at all heterotypic fusion walls and may indicate that intercellular cooperation is needed for the de novo formation of continuous secondary plasmodesmata by fusion of two synchronously built, opposing half plasmodesmal counterparts (Kollmann and Glockmann 1991, 1999; Ehlers and Kollmann 2001). Intraspecific secondary plasmodesmata with different morphotypes have been shown to develop de novo in post-genital fusion walls between coalescing carpel primordia of *Catharanthus roseus* (van der Schoot et al. 1995) and in the contact walls of thyloses extending into xylem-vessel elements (reviewed in Ehlers and Kollmann 2001).

Although unequivocal evidence for a secondary plasmodesmata formation comes from post-genital fusion walls, it has been supposed to be a common event in any growing cell wall of the plant body to compensate for the dilution of eventually existing primary plasmodesmata (e.g., Seagull 1983; reviewed in Ehlers and Kollmann 2001; Roberts 2005; Burch-Smith et al. 2011a). Particularly many secondary plasmodesmata must develop at all the interfaces between clonally unrelated cells which are interconnected by non-division walls undergoing an extreme expansion growth (Ehlers and Kollmann 2001). Such interfaces can be found (1) between the outermost anticlinally dividing tunica layer(s) and the underlying corpus cells in the duplex SAM (van der Schoot and Rinne 1999a, b; Bergmans et al. 1997), (2) between the epidermal layer and the mesophyll in developing leaves (Burch-Smith and Zambryski 2010), (3) between the bundle sheath developing from the ground meristem and the vascular cells derived from the procambial strands in leaves and shoots (see Beebe and Russin 1999), (4) in the radial and tangential walls between longitudinal cell files in the elongation zone of developing roots (Zhu et al. 1998; Rost 2011), and (5) in the radial and transversal walls between radial arrays of cell derivatives in the cambial zone (Ehlers and van Bel 2010; Fuchs et al. 2010a, 2011; Fig. 2.6). In the latter system, de novo formation of complex branched secondary plasmodesmata at locally thinned wall areas seems to play a minor role as complex plasmodesmal morphotypes are lacking at least in the younger developmental stages of the differentiating cambial derivatives. Most additional cell connections seem to develop from plasmodesmal fission, as indicated by the abundance of twinned simple cell connections, and the occasional occurrence of X-shaped and H-shaped intermediates (Ehlers and van Bel 2010; Fuchs et al. 2010a; 2011). Similar observations have also been made for the inner periclinal walls of the epidermis cells in sink leaves of *Nicotiana benthamiana* (Burch-Smith and Zambryski 2010) and may hold true for the other non-division wall interfaces mentioned above. However, complex branched plasmodesmal morphotypes may be formed as the cells undergo their final maturation (Fig. 2.3f–h), as it seems to be a general rule that the degree of plasmodesmal branching increases with the developmental stage of the tissue (e.g., Seagull 1983; Barnett 1987; Ding et al. 1992b; Itaya et al. 1998; Zhu et al. 1998; Oparka et al. 1999; Ormenese et al. 2000; Roberts et al. 2001; Burch-Smith et al. 2011a; Zambryski et al. 2012). The structural changes may be accompanied by functional modifications of the cell connections (e.g., Oparka et al. 1999; Roberts et al. 2001; Kim et al. 2005a, b).

2.4.4 *Loss of Plasmodesmata*

Loss of existing plasmodesmata seems to be another mechanism to adjust the plasmodesmal numbers to changing developmental requirements. The cell connections between immature stomata guard cells and the neighboring epidermal cells are truncated by the deposition of cell-wall material onto the plasmodesmal pore, and they disappear as the cells undergo their final differentiation (Willmer and Sexton 1979; Wille and Lucas 1984). Thus, the highly specialized, mature stomata apparatus becomes symplasmically isolated to allow the turgor regulation of the stomatal pore aperture by targeted ion fluxes across the guard-cell membranes. The same developmentally programmed loss of plasmodesmata has been discussed for the cell connections between immature sieve elements and adjacent phloem parenchyma cells in the transport phloem, since mature sieve-tube members possess extremely low numbers of plasmodesmata in their lateral walls (Gunning 1978; Esau and Thorsch 1985; van Bel and van Rijen 1994; Kempers et al. 1998; van Bel and Ehlers 2000; Hafke et al. 2005), except for the interface(s) to the companion cells where specialized pore/plasmodesma units are formed (e.g., Kempers and van Bel 1997). While the intimate symplasmic connectivity between mature sieve elements and companion cells is required to keep the anucleate sieve elements alive, the symplasmic isolation from the parenchyma cells prevents excessive leakage of assimilates along the phloem pathway (van Bel and Ehlers 2000; Hafke et al. 2005). Moreover, the drastic reduction of the number of simple plasmodesmata occurring in the course of the sink/source transition of tobacco leaves is only partly due to the transformation of simple to branched plasmodesmal morphotypes (Oparka et al. 1999; Roberts et al. 2001; Figs. 2.3b–k and 2.4). In addition, there is a degradation of simple plasmodesmata taking place at the sites where intercellulary spaces are formed in the developing leaves (Roberts et al. 2001). The same event happens in the developing cortex of *Azolla* roots (Gunning 1978). For *Arabidopsis* roots, it has been reported that the reduction of plasmodesmal numbers in the maturing transverse walls clearly exceeds the rate of cell-wall expansion. This most likely indicates the targeted deletion of existing primary plasmodesmata (Zhu et al. 1998). Massive plasmodesmal deletion has also been observed with the older differentiating cell derivatives in the active cambial zone of *Populus nigra* (Fuchs et al. 2010a; 2011; Fig. 2.6). As plasmodesmal deletion at tangential interfaces exceeds that at radial interfaces, the degradation process seems to be highly controlled (Fuchs et al. 2010a; Fig. 2.6). During floral induction in the SAM of *Iris xiphium*, the plasmodesmal densities (number of plasmodesmata per μm^2 cell interface) decrease clearly at all interfaces of the L2 layer (Bergmans et al. 1997). However, it remains unclear whether this change is actually due to the deletion of existing plasmodesmata in the L2-cell walls or to the induced downregulation of primary and/or secondary plasmodesmata formation in the growing and expanding meristem cell layer. A progressive downregulation of the plasmodesmal production was observed during sequential cell divisions of the *Azolla* root initial (Gunning 1978). In growing fern gametophytes, there is an increasing dilution of existing cell connections during cell-expansion growth which

is not compensated by secondary plasmodesmata formation (Tilney et al. 1990; cf. also Imaichi and Hiratsuka 2007). Both mechanisms result in a progressive "loss" of plasmodesmata which was held responsible for the cessation of growth.

Reports on the targeted removal of plasmodesmata during plant development are scarce, possibly because the deletion process is so fast that intermediate stages have seldom been observed (Roberts et al. 2001; Fuchs et al. 2010a). Yet, plasmodesmal deletion was successfully studied in protoplast-derived cell cultures, which form half branched outer-wall plasmodesmata directed to the culture medium during reconstruction of the digested cell walls immediately after protoplast isolation (Monzer 1991; Ehlers and Kollmann 1996, 2001). As shown for the de novo formation of secondary plasmodesmata (Fig. 2.3i), the development of outer-wall plasmodesmata is initiated by the attachment of cytoplasmic ER cisternae to the plasma membrane (Monzer 1991; Ehlers and Kollmann 1996, 2001). Half outer-wall plasmodesmata of neighboring cells may fuse to form a continuous, complex branched cell connection (Monzer 1991; Ehlers and Kollmann 1996, 2001), but mismatching outer-wall plasmodesmata without appropriate counterparts are subjected to the targeted ubiquitination of plasmodesmal proteins and to the selective deletion in the course of cell growth (Ehlers et al. 1996; Ehlers and Kollmann 2001). Following the inverse course of the events observed with the mode of plasmodesmal formation (Fig. 2.3i–k), the outer-wall plasmodesmata are deformed during wall loosening and wall expansion, and they are reintegrated into the cytoplasm by fusion of the plasmodesmal membranes with the underlying plasma membrane (Ehlers et al. 1996; Ehlers and Kollmann 2001).

Summarizing the present paragraph, it can be stated that there are two general rules applying to all mechanisms promoting changes in the plasmodesmal numbers and in the plasmodesmal morphology (Kollmann and Glockmann 1991, 1999; Ehlers and Kollmann 2001; Roberts 2005; Ehlers and van Bel 2010; Figs. 2.3 and 2.4). They all seem to require a locally restricted loosening and/or thinning of preexisting walls which are subsequently rebuilt. Further, the shape of the ER strands incorporated into (or released from) the plasmodesmata seems to determine the plasmodesmal structure.

2.5 Control of Plasmodesmata Formation, Modification, and Deletion

2.5.1 Exogenous and Endogenous Stimuli Controlling Plasmodesmal Formation, Modification, and Deletion

Several studies indicate that there is a precise control of the formation, modification, and deletion of primary and secondary plasmodesmata in the course of developmental processes (e.g., Gunning 1978; Schnepf and Sych 1983; Tilney et al. 1990; Bergmans et al. 1997; Oparka et al. 1999; Roberts et al. 2001; Ehlers and van Bel

2010; Fuchs et al. 2010a, 2011; for reviews, see Ehlers and van Bel 1999; Ehlers and Kollmann 2001; Roberts 2005; Burch-Smith et al. 2011a), but little is known on how the cells exert control on these processes. Exogenous and endogenous stimuli have often been discussed to have an impact on the functional capacities of the cell connections (reviewed in Schulz 1999; Holdaway-Clarke 2005; Benitez-Alfonso et al. 2011; see Chap. 3), and plasmodesmal SELs have been shown to respond at least temporarily, e.g., (1) to cold treatment (Holdaway-Clarke et al. 2000; Rinne et al. 2001, 2011; Sokołowska and Zagórska-Marek 2007; Bilska and Sowiński 2010), (2) to the photoperiod (Rinne and van der Schoot 1998; Gisel et al. 1999, 2002; Ormenese et al. 2002; Ruonala et al. 2008; Fuchs et al. 2010a, 2010b), (3) to pressure gradients (Oparka and Prior 1992), (4) to the application of the gibberellin GA_4 (Rinne et al. 2011), (5) to treatments with sodium azide and N_2 inducing anaerobic stress and a reduction of the cytosolic ATP levels (Tucker 1993; Cleland et al. 1994; Christensen et al. 2009), (6) to H_2O_2 and to the aromatic amino acid tryptophan (Rutschow et al. 2011), (7) to Ca^{2+} (e.g., Holdaway-Clarke et al. 2000; Holdaway-Clarke 2005; Benitez-Alfonso et al. 2011), and (8) to inositol bisphosphate and inositol trisphosphate (Tucker 1988).

Structural alterations of plasmodesmata have only rarely been described. They may occur in response to exogenous changes which have an impact on the physiological state and/or the morphogenetic fate of the tissues. Extracellular callose sphincters constricting the plasmodesmata in dormant buds of *Betula pubescens* (Rinne and van der Schoot 1998) and hybrid aspen (*Populus tremula* × *P. tremuloides*, Ruonala et al. 2008) are degraded after chilling, which induces release of the SAM from dormancy (Rinne et al. 2001, 2011). Moderate osmotic stress applied to the roots of *Pisum sativum* causes an increase in the size of the plasmodesmal cytoplasmic sleeves and an enhanced unloading of the phloem (Schulz 1995). In leaves of a chilling-sensitive maize line showing decreased photosynthetic activity and reduced assimilate export when exposed to suboptimal temperatures, the lumen of the plasmodesmal cytoplasmic sleeves is reduced between bundle sheath and vascular parenchyma cells, and internal sphincters constrict the cell connections at the bundle sheath/Kranz mesophyll interface (Bilska and Sowiński 2010). Remarkably, plasmodesmal densities (number of plasmodesmata per micrometer vein) increase significantly at the same cell interfaces in response to the moderate chilling, and this response was slightly lower in the chilling-sensitive than in a chilling-tolerant maize line (Sowiński et al. 2003; cf. Chaps. 1 and 3).

As compared to temporary functional modifications and minor structural alterations, changes in the plasmodesmal numbers may represent a long-lasting mechanism by which plasmodesmal networks can be adapted to altered exogenous conditions. In maize and other C4 grasses, plasmodesmal densities (number of plasmodesmata per micrometer vein) also rise with increasing light intensities at the bundle sheath/Kranz mesophyll interface as well as at the bundle sheath/vascular parenchyma interface. Thus, transport capacities are possibly adjusted to the increasing production of photosynthates by formation of additional plasmodesmata (Sowiński et al. 2007). Floral induction by exposure to long-day regime (Ormenese et al. 2000), as well as cytokinin application (Ormenese et al. 2006), promotes a

threefold increase of the plasmodesmal densities (number of plasmodesmata per µm wall length) at the anticlinal and periclinal interfaces of all initial layers in the SAM of *Sinapis alba* which indicates an increase in secondary plasmodesmata formation. Photoperiodic flower induction in the SAMs of the short-day plant *Perilla nankinensis* and the long-day plant *Rudbeckia bicolor* (Milyaeva 2007) is also accompanied by an increase in the plasmodesmal densities (number of plasmodesmata per µm wall length) at most interfaces in the central zone which produces the reproductive organs, except for the anticlinal wall between the L1 and L2. In contrast, plasmodesmal densities are reduced in the medullar zone giving rise to the stem tissues (Milyaeva 2007). As mentioned above, floral induction causes selectively decreasing plasmodesmal densities (number of plasmodesmata per μm^2 cell interface) in the L2-layer of the SAM of *Iris xiphium*, so that the symplasmic connectivity of the L1 to the meristem corpus becomes reduced (Bergmans et al. 1997). Moreover, plasmodesmal densities (number of plasmodesmata per μm^2 wall) and total numbers of plasmodesmata undergo drastic seasonal changes in the cambial zone of *Populus nigra*, so that plasmodesmal connectivity is high in the active state and low in the dormant state of these stem tissues (Fuchs et al. 2010a; see Fig. 2.6 below).

2.5.2 Molecular Mechanisms Controlling Plasmodesmal Formation, Modification, and Deletion

2.5.2.1 Redox Regulation

New hints on the molecular mechanisms controlling the formation and modification of primary and secondary cell connections come from recent analyses on *Arabidopsis* mutants which show altered plasmodesmal transport capacities during embryogenesis. These studies point at a redox regulation of the symplasmic transport pathways (reviewed in Benitez-Alfonso et al. 2011; Burch-Smith et al. 2011a, b; Burch-Smith and Zambryski 2012; Zambryski et al. 2012). Enhanced callose deposition at plasmodesmata, particularly in the RAM, occurs in the seedling lethal *Arabidopsis* mutant *gat1* (*green fluorescent protein arrested trafficking*; Benitez-Alfonso et al. 2009). The *gat1* mutant is defective in the plastidial thioredoxin m-type3 gene which is involved in controlling the cellular redox state and the homeostasis of reactive oxygen species (ROS). The increased ROS level in *gat1* was hypothesized to induce callose synthesis presumably mediated by the elevation of the intracellular Ca^{2+} level (Holdaway-Clarke et al. 2000; Holdaway-Clarke 2005; Benitez-Alfonso et al. 2011). This, in turn, reduces the plasmodesmal SEL and stops the unloading of GFP from the phloem (Benitez-Alfonso et al. 2009). Increased ROS levels were also found in the embryo lethal *Arabidopsis* mutant *ise1* (*increased size exclusion limit*, Stonebloom et al. 2009, 2012). However, *ise1* has an increased plasmodesmal SEL.

These contradictory results might be explained by different amounts of ROS production in *gat1* vs. *ise1*. Low ROS levels may increase the plasmodesmal SEL, while

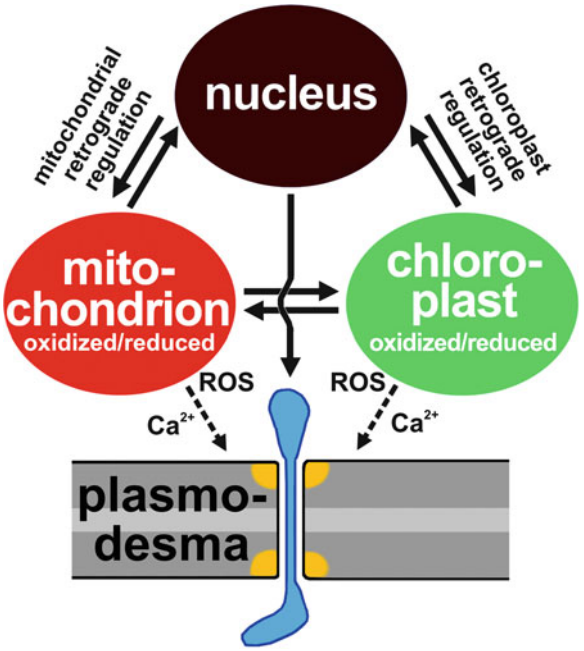

Fig. 2.5 Model of the organelle-nucleus-plasmodesmata-signaling pathway [as suggested by Burch-Smith et al. (2011b) and Burch-Smith and Zambryski (2012)]. Chloroplasts and mitochondria exchange signals with each other and with the nucleus to induce transcriptional regulation of plastidial and mitochondrial genes in order to adjust the organelle metabolism to changes in the energy status and in the redox state within the cell. The regulatory network also controls structural and functional alterations of the plasmodesmata. Whether mitochondrial and plastidial signals exert direct control on plasmodesmata is not known (*dashed arrows*). Changes in the intercellular levels of ROS and/or Ca^{2+} may function in such a signaling pathway, e.g., to modify the callose deposits at the plasmodesmal orifices (*yellow*) which in turn regulate the plasmodesmal size exclusion limit (Benitez-Alfonso et al. 2011). Nuclear regulation comprises the differential expression of genes coding for plasmodesmal proteins, and the adjustment of transcript levels of enzymes involved in cell-wall formation and modification. The latter may be required for the cell-wall remodeling during plasmodesmal formation and modification

high ROS concentrations may cause plasmodesmal occlusion (Rutschow et al. 2011). Alternatively, plasmodesmata may be part of an intricate organelle-nucleus-plasmodesmata-signaling pathway (ONPS, Burch-Smith et al. 2011b; Burch-Smith and Zambryski 2012; Fig. 2.5), in which the metabolism of the organelles is interconnected via the energy status and the modulation of cellular redox state (Burch-Smith et al. 2011b; Burch-Smith and Zambryski 2012; Stonebloom et al. 2012). Thus, it may be critical for the plasmodesmal response, in which organelle the ROS production takes place (Burch-Smith and Zambryski 2012; Stonebloom et al. 2012). In *gat1*, ROS production results in an oxidized state in the plastids, which induces a reduced plasmodesmal SEL, while ROS production causes oxidized mitochondria in *ise1* due to a defective mitochondria-localized DEAD-box RNA helicase gene.

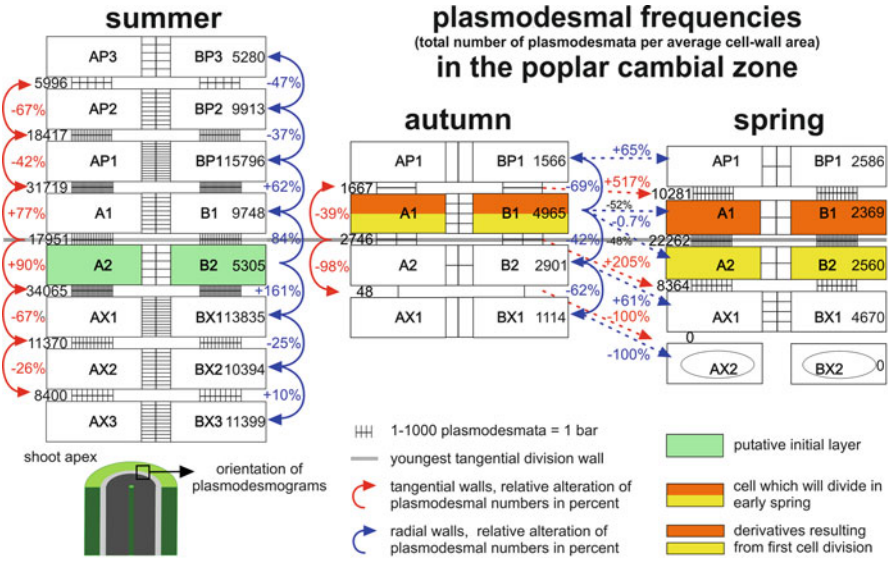

Fig. 2.6 Plasmodesmograms of the cambial zone of *Populus nigra* in three seasonal stages. The cambial plasmodesmal networks as viewed in cross sections through the 2–3-year-old twigs are depicted (1) in the active state in summer (*left*), (2) in the dormant state in autumn (*middle*), and (3) during release from dormancy (budbreak) in spring (*right*). The *gray line* connecting the plasmodesmograms marks the respective youngest tangential division wall. *Rectangles* labeled with A1 and B1 represent the phloem-sided cells bordering this division wall in two adjacent radial cell rows. The radially aligned phloem-sided cell derivatives were further coded successively according to the chronological order of their formation, starting with the youngest derivative as AP1, AP2, etc. (or BP1, BP2, etc.). *Rectangles* labeled with A2 and B2 depict the cells which directly border on the youngest division wall at the xylem-side; the following xylem-sided derivatives were successively coded as AX1, AX2, etc. (or BX1, BX2, etc.). Tangential and radial cell walls were named after the respective adjoining cells, e.g., A1/AP1 or A2/B2. Absolute plasmodesmal frequencies were calculated as total number of plasmodesmata per total interface area (computed as average cell-wall area). They are indicated by *black numbers* and by the *bars* between the rectangles (1,000 plasmodesmata represent 1 bar). Due to regular cell-division patterns, the topological sequence of cambial cell interfaces mirrors the cell-wall development in chronological order, and plasmodesmal development can be deduced by analyzing the consecutive walls at the phloem-side and xylem-side (see Ehlers and van Bel 2010). Changes in the plasmodesmal frequencies in successive tangential division walls are indicated by *red arrows* and *numbers*, *blue arrows* and *numbers* indicate relative changes in plasmodesmal frequencies in radial non-division walls. *Green color* marks the putative initial layer in summer, *orange* and *yellow colors* indicate the cells involved in the first cell division in spring. In the active state in summer, plasmodesmal frequencies undergo dynamic changes depending on the developmental state of the derivatives in the numerous layers of the cambial zone. Starting from relatively low numbers in the youngest tangential division walls (A1/A2), there is a drastic increase in the plasmodesmal numbers by +77 % on the phloem-side (A1/A1P) and by +90 % on the xylem-side (A2/AX1). This increase is presumably performed by plasmodesmal fission, as indicated by the abundance of twinned plasmodesmata and by the occurrence of intermediate branched morphotypes (cf. Fig. 2.4l). The resulting high plasmodesmal frequencies may build up a preferential pathway for the transfer of tissue-specific positional signals to the youngest cambial derivatives to determine their xylem or phloem fate. During further cell differentiation, plasmodesmal numbers are subsequently reduced by plasmodesmal deletion in the tangential walls. Note that the oldest cell derivatives which were observed, had not

Plastids were in the reduced state in *ise1* and also in the *Arabidopsis* mutant *ise2* (Kobayashi et al. 2007; Burch-Smith and Zambryski 2010), which is defective in a chloroplast localized DEVH box RNA helicase. Consequently, the plasmodesmal SEL is increased in both *ise* mutants (Stonebloom et al. 2012; Burch-Smith and Zambryski 2012).

Remarkably, the mutant analyses suggest that the redox regulation also affects plasmodesmal structure. In *gat1* mutants, 5 % of the plasmodesmata were found to be occluded (Benitez-Alfonso et al. 2009; Xu et al. 2012), but early reports on a higher degree of plasmodesmal branching in this mutant (Benitez-Alfonso et al. 2009) have been emended later on (Burch-Smith et al. 2011a; Zambryski et al. 2012). However, as compared to the hypocotyls of wild-type embryos of the

Fig. 2.6 (continued) reached the mature state. Similar events occur at the radial interfaces starting from the radial non-division wall with the lowest plasmodesmal frequency (A2/B2) which most likely marks the initial layer. The initial increase of plasmodesmal frequencies by plasmodesmal fission on the phloem-side (A1/B1, +84 %; AP1/BP1, +62 %) and on the xylem-side (AX1/BX1, +161 %) may mediate tangential exchange of cell-fate-specifying signals across the radial interfaces, once the tissue identity is established by radial signaling across the tangential interfaces [A1/A1P, A2/AX1; cf. also Fuchs et al. (2011)]. Later on, a deletion of plasmodesmata takes place, but the deletion process at the radial interfaces is not as fast as at the tangential interfaces, indicating a precise tuning of the plasmodesmal numbers in the course of cell development. The plasmodesmal networks are also adapted to seasonal changes. In autumn, the cambial zone comprises only a few layers of immature derivatives which exhibit fragmented vacuoles filled with storage materials and cryoprotectants. These cytological features are typical for the dormant state (Lachaud et al. 1999; Fuchs et al. 2010b). As compared to the active state, plasmodesmal numbers are extremely low at all interfaces of the dormant cambial zone, except for the radial wall A1/B1. Here, the total number of 4,965 plasmodesmata per average cell-wall area resembles that of the radial interface between active cambial initials in summer (*green color*, A2/B2, 5305 plasmodesmata per average cell-wall area). Interestingly, the relatively high plasmodesmal numbers mark the layer of the dormant cambial initials (A1, B1, etc.). Unchanged plasmodesmal frequencies at their radial interfaces may allow immediate symplasmic communication to coordinate the first synchronous tangential cell division in spring (*orange* and *yellow color*). To the best of our knowledge, the unchanged plasmodesmal frequency is the only cytological marker which characterizes the cambial initials. The first tangential division wall (A1/A2) laid down in spring is already equipped with 22,262 plasmodesmata per average cell-wall area, which is comparable to the high frequency observed in the respective wall in summer (A1/A2, 17,951 plasmodesmata per average cell-wall area). At the radial interfaces of the two cell layers resulting from this first division (A1/B1 and A2/B2), plasmodesmal numbers have not increased, but the preexisting plasmodesmata at the autumnal interface have been distributed in almost identical numbers over the splitted wall halves (−52 % and −48 %, respectively; total: −0.7 %; relative changes are indicated by the *dotted lines*). As compared to the dormant state, however, plasmodesmal frequencies rise clearly in other tangential and radial walls which had been laid down before dormancy (*dotted lines*), i.e., with the precursor phloem (AP1, BP1, etc.) and with the boundary parenchyma (AX1, BX1, etc.). In contrast, no plasmodesmata were observed at the interfaces of the cell layer AX2, BX2, etc. During cambial reactivation, these cells have rapidly developed into xylem vessels from the oldest xylem-sided derivatives of the previous year. Thus, they have lost all their plasmodesmata during maturation [reprinted from Fuchs et al. (2010a), by permission of Oxford University Press]

late-torpedo stage (9 %), higher relative fractions of branched and twinned plasmodesmata were found in *ise1* (26–28 %) and *ise2* (13–15 %). This indicates increased formation of secondary plasmodesmata (Burch-Smith and Zambryski 2010; Burch-Smith et al. 2011a; Zambryski et al. 2012) most likely by promoting plasmodesmal fission (Ehlers et al. 2004; Faulkner et al. 2008; Ehlers and van Bel 2010). In contrast, decreased relative fractions of branched and twinned plasmodesmata (6.6 %) and concomitant reduced plasmodesmal SELs were recently found in the *Arabidopsis* mutant *dse1* (*decreased size exclusion limit*, Xu et al. 2012). This mutant is defective in the WD-repeat protein TANMEI which localizes to the cytoplasm and the nucleus.

As *ISE1*, *ISE2*, and *DSE1* do not localize to plasmodesmata, their impact on plasmodesmal formation and plasmodesmal structure must be indirect (Burch-Smith et al. 2011a; Burch-Smith and Zambryski 2012; Xu et al. 2012; Zambryski et al. 2012). Whole-genome microarray analyses of the *ise1* and *ise2* mutants demonstrated alterations in the transcript levels of a broad variety of genes, which was attributed to a ROS-induced organelle-nucleus-plasmodesmata signaling exerting control on the gene expression patterns (Burch-Smith et al. 2011b; Fig. 2.5). Besides gene products involved in plastidial and mitochondrial function, several plasmodesmal proteins were differentially expressed in *ise1*, *ise2*, and wild types. Moreover, enzymes involved in cell-wall formation and modification were affected (Burch-Smith et al. 2011b; Fig. 2.5), and these may play a role in the cell-wall remodeling required for plasmodesmal formation, modification, and deletion (e.g., Kollmann and Glockmann 1999; Ehlers and Kollmann 2001; Roberts 2005). Burch-Smith et al. (2011b) reported that both *ise1* and *ise2* showed a transcriptional upregulation of cellulose synthases and cellulose synthase-like proteins involved in the production of cross-linking glycans (hemicelluloses), a massive upregulation of xyloglucan-endotransglucosylases/hydrolases with a probable function in wall-expansion growth, and a downregulation of expansins which mediate acid-induced cell-wall expansion (e.g., Cosgrove 2005).

ROS may also have a direct effect and may provoke an immediate, nonenzymatic cell-wall loosening which facilitates the structural dynamics of the cell connections. It has been suggested that endogenous hydroxyl radicals ($^{\bullet}OH$) are generated by class III cell-wall peroxidases from superoxide and H_2O_2, which in turn is produced by a NAD(P)H oxidase in the plasma membrane from the reduction of monovalent O_2 (Liszkay et al. 2003). The highly active $^{\bullet}OH$ may function as a wall-loosening agent, as it can cleave cell-wall polymers in an unspecific manner (Schweikert et al. 2000). The general role of $^{\bullet}OH$ in wall-extension growth suggested by Schopfer et al. (Schopfer et al. 2002; Liszkay et al. 2003) has been challenged, because the $^{\bullet}OH$ amount needs to be low to avoid damage of the living cells (Cosgrove 2005; Schopfer 2006). Further, $^{\bullet}OH$ can be expected to operate only in the immediate vicinity of its production site, due to its short lifetime of a few nanoseconds. Just this feature, however, characterizes $^{\bullet}OH$ as an attractive candidate agent for the rapid and locally restricted wall modifications needed in the course of plasmodesmal development. Two components, required for the formation of $^{\bullet}OH$ have recently been detected in the cell walls of the cambial zone of tomato stems, where a dynamic regulation of plasmodesmal connectivity takes place (Ehlers and van Bel 2010).

Immunolocalization confirmed the occurrence of peroxidases associated with the cambial plasmodesmata. Moreover, the presence of H_2O_2 was detected cytochemically by its reaction with $CeCl_3$, resulting in a punctate distribution of cerium-perhydroxide precipitates in the cambial walls, which were frequently associated with plasmodesmata (Ehlers and van Bel 2010). Molecular data support the presence of class III peroxidases in the *Arabidopsis* plasmodesmal proteome, but the localization has not yet been proven (Fernandez-Calvino et al. 2011).

2.5.2.2 Membrane Domains

Plasmodesmal deployment seems to be precisely programmed during plant development, but it still remains unclear, how the sites of primary and secondary plasmodesmal formation are selected by the cells. We speculate that local peculiarities of the plasma membrane may play a crucial role, e.g., remorin-containing lipid rafts (Raffaele et al. 2009; Mongrand et al. 2010; Tilsner et al. 2011; Blachutzik et al. 2012; Perraki et al. 2012; see Tanner et al. 2011) or other microdomains corralled by transmembrane proteins which are tightly connected to the cytoskeleton and/or to the cell-wall components. These connections limit the lateral diffusion of the membrane components which can only escape from the corralled microdomains by "hop diffusion" (Tilsner et al. 2011; Martinière et al. 2012). The membrane domains may contain or attract aggregates of proteins involved in the local loosening and/or thinning of eventually preexisting cell walls at the extracellular side, e.g., xyloglucan-endotransglucosylases, expansins, or NAD(P)H oxidases. Further, the microdomains may either occur within or close by the plasmodesmal-associated parts of the membrane to mediate modifications of existing cell connections, plasmodesmal fission, and plasmodesmal degradation (Raffaele et al. 2009; Mongrand et al. 2010; Figs. 2.3e–h and 2.4l) or they may be scattered over the cell surface and initiate de novo plasmodesmata formation (Mongrand et al. 2010; Fig. 2.3i).

Intracellularly, the same microdomains may enable the targeted attachment of cytoplasmic ER strands to the plasma membrane, in order to initiate plasmodesmata modification, fission, or de novo formation driven by cell-wall (re)construction (Figs. 2.3b–d, i–k and 2.4l). Alternatively, the ER attachment may be mediated by ER-localized membrane domains (see Tilsner et al. 2011), and it is even possible that it is the attachment of the ER which actually initiates the formation of microdomains with wall-modifying enzymes in the plasma membrane. Already during cytokinesis, microdomains may mediate the attraction of the cytoplasmic ER strands to the membrane surrounding the growing cell plate, which represents the future plasma membrane (Fig. 2.3b). This may determine the sites of primary plasmodesmata formation. It has been shown in *Arabidopsis* root tips that the membrane at the growing margins of the cell plate has a special protein equipment, as it belongs to the post-Golgi membrane domain characterized by Rab-A2/Rab-A3 GTPases which are known to function in vesicle trafficking (Chow et al. 2008; Qi and Zheng 2012).

Moreover, it also points to a possible contribution of membrane microdomains that isolated plant protoplasts start immediately with the formation of outer-wall

plasmodesmata in the course of cell-wall reconstruction (Monzer 1991; Ehlers and Kollmann 1996, 2001). Conceivably, the plasmodesmal formation takes place at those sites, where the cells have had cell connections within the intact tissue. As these cell connections had suddenly been ripped apart during protoplast isolation, the former plasmodesmal membrane domains may have been preserved in the plasma membrane to initiate rapid plasmodesmal reconstruction. Protoplasts may be an ideal system to test this hypothesis. In contrast, targeted deletion of plasmodesmata seems to be accompanied by the selective degradation of ubiquitinated proteins (Ehlers et al. 1996), which may possibly destroy the plasmodesmal membrane domains.

2.5.2.3 ER Shaping

It has often been pointed out that the ER plays a crucial role in plasmodesmal development—not only because it may determine the sites of primary (and secondary?) plasmodesmal formation but also because the plasmodesmal morphology seems to depend directly on the shape of the ER strands which are transformed into the plasmodesmal desmotubules (see Kollmann and Glockmann 1999; Ehlers and Kollmann 2001; Roberts 2005; Ehlers and van Bel 2010; Burch-Smith et al. 2011a; Tilsner et al. 2011; see Figs. 2.3 and 2.4). Thus, it is reasonable to speculate that those mechanisms which shape the cortical ER network within the plant cells may have a direct impact on the plasmodesmal numbers and on the plasmodesmal morphology (Faulkner et al. 2008; Ehlers and van Bel 2010; Tilsner et al. 2011).

It is well known that the cellular ER network is highly dynamic. It moves along cytoskeleton filaments—i.e., microtubules in animals and actin filaments in yeast and plants—and it undergoes a continuous, but controlled, remodeling (Staehelin 1997; Boevink et al. 1998; Prinz et al. 2000; Sparkes et al. 2010; Ueda et al. 2010; Hu et al. 2011; Pendin et al. 2011; Tilsner et al. 2011; Chen et al. 2012; Lin et al. 2012). Two morphologically distinct forms can be distinguished in the ER network, which presumably also differ with respect to their functional tasks. Flat, fenestrated ER sheets, which may be studded with ribosomes, are found besides smooth, interconnected, curved tubules, a subdomain of which is the transitional ER involved in the secretory pathway (Staehelin 1997; Hu et al. 2011; Pendin et al. 2011; Chen et al. 2012; Lin et al. 2012; Puhka et al. 2012). The two ER forms are contiguous and can be transformed into each other, presumably via intermediate stages with a fenestrated morphology (Puhka et al. 2012). The relative amounts of the ER forms vary between different cell types (Hu et al. 2011; Lin et al. 2012), with the developmental stage of the cells (Ridge et al. 1999; Chen et al. 2012), and during cell division (Chen et al. 2012; Puhka et al. 2012). Great progress has been made in the past years in understanding the molecular mechanisms which govern ER dynamics. However, most information comes from studies on animal cells and yeast.

Two ubiquitous protein families of eukaryotes have been identified as membrane curvature-stabilizing proteins, which are supposed to shape the ER tubules and the edges of the ER sheets (Voeltz et al. 2006; Hu et al. 2008; Shibata et al. 2010) and which may also be involved in the formation of the tightly curved desmotubule

membranes (Tilsner et al. 2011). These proteins are the reticulons—like RTN4a/NogoA in mammals and RTN4 in yeast (Voeltz et al. 2006)—and a protein family of reticulon interaction partners represented by DP1/REEP5 in mammals and Yop1p in yeast (deleted in polyposis 1/YIP (Ypt interacting protein) one partner 1; Voeltz et al. 2006; Hu et al. 2008, 2011). Although they do not share sequence homologies, reticulons and DP1/Yop1p family members are characterized by two hydrophobic domains forming hairpin loops which expand the outer leaflet of the ER membrane to induce curvature. At the cytoplasmic side, the proteins interact with each other to form oligomers which may scaffold the curved membrane (Hu et al. 2011; Pendin et al. 2011; Tilsner 2011; Chen et al. 2012; Lin et al. 2012). Twenty-one reticulon-like proteins (RTNLB1–21) have been identified in *Arabidopsis*, five of which have been shown to be involved in ER-membrane shaping (Sparkes et al. 2010; Tolley et al. 2010). Moreover, Chen et al. (2009, 2011, 2012) described HVA22 proteins in *Hordeum vulgare* and *Arabidopsis thaliana* as homologs of DP1/Yop1p, but their involvement in ER-tubule formation has not been proven.

CLIMP63 was supposed to be a special mammalian transmembrane protein contributing to ER-sheet formation, as it may form scaffolds on the luminal side of the ER membrane (Shibata et al. 2010; Hu et al. 2011; Tilsner et al. 2011; Lin et al. 2012). CLIMP63 and other proteins of the ER membranes, like STIM1, p180, and REEP1, have also been held responsible for the association of the ER to the microtubular cytoskeleton network which somehow functions in the ER remodeling (reviewed in Hu et al. 2011; Pendin et al. 2011; Chen et al. 2012; Lin et al. 2012; Puhka et al. 2012). Yet, plant homologs of CLIMP63 have not been identified so far, and the influence of the actin/myosin network on the ER structure in plants has not been studied in detail. Motility of the ER (and Golgi stacks) in plant cells has been shown to depend on the actin/myosin system (Boevink et al. 1998; Ueda et al. 2010; Yokota et al. 2011), and *in vitro*-studies suggest that myosin XI drives ER-tubule formation along actin filaments (Yokota et al. 2011; see Chen et al. 2012). In yeast, ER dynamics decreases after depolymerization of actin filaments, but the ER network does not collapse, as it was shown for mammalian cells after microtubule depolymerization (Prinz et al. 2000; see Hu et al. 2011; Lin et al. 2012). Possible interactions of the ER and the actin/myosin network (Boevink et al. 1998; Ueda et al. 2010; Yokota et al. 2011; Chen et al. 2012; Tilsner et al. 2012) may be of particular interest for the plasmodesmal research, since the actin/myosin network has often been discussed to be an important structural component of plasmodesmata which has an impact on the functional plasmodesmal properties including the control of viral transport (for reviews, see, e.g., Faulkner and Maule 2011; Fernandez-Calvino et al. 2011; Niehl and Heinlein 2011; Radford and White 2011; Schoelz et al. 2011; Tilsner et al. 2011, 2012). In this context, special attention should be paid to the members of the recently identified Networked (NET) protein superfamily functioning as plant-specific actin/membrane connectors (Deeks et al. 2012). In *Arabidopsis*, this family of membrane-associated proteins, which is characterized by a special actin-binding domain, comprises thirteen members belonging to four phylogenetic clades with specific subcellular localizations (Deeks et al. 2012). NET1A has been shown to localize to the plasma membrane and to plasmodesmata to allow actin/membrane interactions (Deeks et al. 2012).

With respect to the possible role of the peripheral ER in structural plasmodesmal development, it is not sufficient to know how the ER membranes are shaped into flat sheets or curved tubules and how these structures interact with the cytoskeleton. It is also interesting to investigate how the branching of the ER tubules is achieved and controlled. In metazoans, this function has been attributed to atlastins (ATLs), a class of dynamin-like GTPases which mediate the homotypic fusion (and/or fission) of ER tubules, in order to insert (and/or remove) branching points in the tubular ER network (Hu et al. 2009, 2011; Orso et al. 2009; Pendin et al. 2011; Chen et al. 2012; Lin et al. 2012). ATLs are also hairpin-forming transmembrane proteins of the ER and they interact with reticulons and DP1 in the membrane. Analogous proteins with similar functions are the GTPase Sey1p in yeast (Hu et al. 2009, 2011; Anwar et al. 2012) and three isoforms of ROOT HAIR DEFECTIVE 3 (RHD3) with partially redundant function in *Arabidopsis* (Chen et al. 2011; Hu et al. 2011; Lin et al. 2012). RHD3 colocalizes with the above-mentioned HVA22d in a punctate distribution along the ER tubules (Chen et al. 2011; Stefano et al. 2012) which matches the postulated function in local membrane fusions/fissions. Remarkably, RHD3 also has an impact on the motility of Golgi stacks (Chen et al. 2011, 2012; Stefano et al. 2012) which may be of particular interest in view of the role of the Golgi apparatus in cell-wall growth and plasmodesmal development. Moreover, it was mentioned by Chen et al. (2012) that a mutation in *rhd2-1*, coding for a NADPH oxidase responsible for ROS production, is epistatic to *rhd3-1* during root-hair growth in *Arabidopsis* (Schiefelbein and Somerville 1990), which may point to a comprehensive correlation between ER shaping and redox-regulated cell-wall growth. Plasmodesmal formation and/or modification are possible links between these two processes.

In contrast to our detailed image of heterotypic membrane fusion mediated by SNAREs and Rab proteins (Hu et al. 2011; Lin et al. 2012), however, information on the processes of homotypic membrane fusion and membrane fission during ER remodeling in plants is still scarce. It will be interesting to follow the progress made in this research area, since it might have enormous consequences for our understanding of the molecular control of plasmodesmal dynamics, in particular for the structural changes occurring in the plasmodesmal networks of developing plant tissues.

2.6 Discrepancies and Open Questions

2.6.1 Mechanisms Which Cause Alterations of the Plasmodesmal Networks

The mechanisms which alter the plasmodesmal numbers and the structural properties of the plasmodesmal network are discussed extensively in the previous paragraphs. Besides, there are several modes to alter the functional capacities of the cell connections. Callose sphincters, the actin/myosin system, phosphorylation/dephosphorylation of plasmodesmal proteins, and alterations of the plasmodesmal protein

equipment have been held responsible for changes in the plasmodesmal aperture and the size of the plasmodesmal microchannels (e.g., Schulz 1999; Ehlers and Kollmann 2001; Holdaway-Clarke 2005; Benitez-Alfonso et al. 2011; Faulkner and Maule 2011; Deeks et al. 2012). Moreover, there is another mechanism of plasmodesmal regulation which was initially observed with defined stages of developing antheridial filaments of *Chara vulgaris* (for reviews, see Kwiatkowska 1999; Ehlers and Kollmann 2001). Prior to spermatogenesis, the cell connections at particular antheridial interfaces were reversibly occluded with an electron-dense material in order to demarcate distinct symplasmic domains whose cells show different cell cycle activities or have reached different stages of cell differentiation. Within such a domain, however, development of the cells was completely synchronized. A reversible plugging of plasmodesmata controlled by the cytokinin/ABA ratio was also observed in the seed coat and the mesocarp of developing avocado fruits, where it causes seed coat senescence and retardation of fruit growth (Moore-Gordon et al. 1998). Moreover, occluded plasmodesmata were found in protoplast-derived calluses of the dicot *Solanum nigrum* and in globular somatic embryos developed from scutellar calluses of the monocot *Molinia caerulea* (Fig. 2.4a). Thus, it was suggested that plugging of plasmodesmata might be a general event in plant development and a widespread mechanism to establish temporary symplasmic domains within the tissues (Ehlers et al. 1999). However, occluded cell connections have not been observed in the course of the detailed examination of the plasmodesmal development in plant embryos (Burch-Smith and Zambryski 2010; Burch-Smith et al. 2011a; Xu et al. 2012; Zambryski et al. 2012) and sink leaves (e.g., Oparka et al. 1999; Roberts et al. 2001). Only Benitez-Alfonso et al. (2009) reported the occurrence of 5 % "occluded" plasmodesmata in embryos of the *gat* mutant. The pictures presented for the *gat* mutant plasmodesmata are, however, not identical to those of occluded plasmodesmata in the other systems, where the occluding material is located within the microchannels of the cytoplasmic sleeve or within the plasmodesmal orifices (Moore-Gordon et al. 1998; Kwiatkowska 1999; Ehlers et al. 1999), but they rather resemble the plasmodesmata with extracellular sphincters found in dormant shoot apices (Rinne and van der Schoot 1998; Ruonala et al. 2008). Similar sphincter-like structures, interpreted as wall collars, can also be found at some of the plasmodesmata shown in Oparka et al. (1999). However, e.g., the plasmodesmata shown in Figs. 6Aii and 6Bii of Oparka et al. (1999) may actually be interpreted as occluded cell connections. It remains an open question whether plasmodesmal plugging indeed plays a significant role in the developing plant tissues.

2.6.2 *Plasmodesmal Responses to Environmental Stimuli Which Cause Morphogenetic Switches*

Further discrepancies occur in the literature, concerning the plasmodesmal responses to environmental stimuli. It cannot be answered, as yet, whether these discrepancies point to actual differences in the distinct tissues/species used for

the experiments or whether they may (partly) be due to different experimental techniques and calculation methods. A short-day regime has been found to induce dormancy in the SAM of *Betula pubescens* which leads to the symplasmic isolation of the meristem cells (Rinne and van der Schoot 1998). During dormancy, callose sphincters were observed to constrict the plasmodesmata in the SAM of birch (Rinne and van der Schoot 1998) and poplar (Ruonala et al. 2008), but callose degradation and the restoration of the symplasmic organization of the SAM is induced by chilling (Rinne et al. 2001, 2011). In contrast, no plasmodesmal sphincters were found in the dormant cambial zone of poplar in autumn, but the plasmodesmal numbers were drastically reduced (Fuchs et al. 2010a, 2011; Fig. 2.6) and the remaining plasmodesmata were found to be closed, as they do not mediate the transfer of the small, intracellularly injected fluorescent dye Lucifer Yellow CH (Fuchs et al. 2010a, b). Thus, the symplasmic isolation of dormant cambial cells and of dormant cells in the SAM is obviously achieved by different mechanisms. However, studies on twigs of *Acer pseudoplatanus* and *Ulmus minor* did not confirm the symplasmic isolation of the dormant cambial cells (Sokołowska and Zagórska-Marek 2007). In contrast, the authors reported the existence of a symplasmic border at the interface between ray cells and fusiform cells in summer and symplasmic continuity in winter, as tested by application of fluorescent dyes over several days. These results clearly contradict the findings of Fuchs et al. (2010b) who demonstrated the symplasmic transport of a purified 4.4-kDa FITC-labeled dextran fraction between ray cells and fusiform cells during poplar cambial reactivation in spring by using microinjection techniques.

The changes in the plasmodesmal networks which were observed in the SAM after photoperiodic flower induction are also not consistent, as plasmodesmal densities were either reported to increase in the L1–L3 of *Sinapis alba* (Ormenese et al. 2000, 2006) or to decrease in the L2 of *Iris xiphium* (Bergmans et al. 1997). Milyaeva (2007) found tissue-specific differences in the plasmodesmal response of the SAM in *Perilla nankinensis* and *Rudbeckia bicolor*. Increasing plasmodesmal densities occurred in the central zone, except for the anticlinal wall between the L1 and L2, but plasmodesmal densities decreased in the medullar zone. Concurrently, floral induction has been reported to be accompanied by the transient symplasmic isolation of the SAM as indicated by the lack of import of fluorescent tracers (e.g., Gisel et al. 1999, 2002; for a review, see Kobayashi et al. 2005). Comparable results can, however, only be produced, if identical techniques were used for the experimental setup and the data processing. For example, as already criticized by Ehlers and van Bel (1999), there are still too many different ways to compute plasmodesmal densities and frequencies. The best estimation of the actual symplasmic connectivity at a given interface can certainly be based on the absolute plasmodesmal frequencies, i.e., the total number of plasmodesmata between the cells (Ehlers and van Bel 1999; Fig. 2.6).

2.6.3 *Preferential Symplasmic Transport Pathways, Symplasmic Barriers, and the Particular Role of Secondary Plasmodesmata*

The plasmodesmal development of *Arabidopsis* embryos has been studied intensely and the results have been summarized in several recent reviews (e.g., Kobayashi et al. 2005; Burch-Smith et al. 2011a; Zambryski et al. 2012). Up to the early heart stage, the embryo consists of one single symplasm domains with a high plasmodesmal SEL, but at the mid-torpedo stage, the embryo has become separated into distinct symplasmic subdomains whose borders exhibit different plasmodesmal SELs. As the domains correspond to the basic organs of the embryo, i.e., cotyledons, SAM, hypocotyl, and root (Kim et al. 2005a, b), the plasmodesmal transport capacities seem to concur with morphogenetic events. Similarly, a drastic reduction of the plasmodesmal SEL from approximately 50 kDa (GFP fusions) to less than 27 kDa (GFP) and the concomitant transformation of simple into branched plasmodesmal morphotypes corresponds exactly with the sink-source transition of developing leaves (Oparka et al. 1999; Roberts et al. 2001). In the cambial zone of poplar and of the herbaceous plants tomato and *Arabidopsis*, particularly high numbers of plasmodesmata mark the predicted preferential pathways for the symplasmic exchange of positional information to determine tissue and cell fate (Ehlers and van Bel 2010; Fuchs et al. 2010a, 2011; Fig. 2.6). These preferential pathways are established by massive formation of "twinned secondary plasmodesmata" in the division and non-division walls.

In contrast, in *Arabidopsis* root tips, plasmodesmal densities are low in the tangential non-division walls between tissue cylinders, while high plasmodesmal densities occur in the transverse division walls within cell files (Zhu et al. 1998; Zhu and Rost 2000; Rost 2011). The plasmodesmal distribution largely corresponds with functional studies, which demonstrated predominantly acropetal spread of fluorescent dyes which were symplasmically unloaded from the protophloem to the RAM (Oparka et al. 1994). Unhindered post-phloem transport occurred with GFP (27 kDa), while transport of larger GFP fusions (up to 67 kDa) was restricted to the cells that directly adjoin to the protophloem (Stadler et al. 2005). However, as discussed above (Fig. 2.2), the tangential interfaces within the developing root are particularly important for the symplasmic exchange of morphogenetic signals, like transcription factors and sRNA species, to determine tissue patterning (Furuta et al. 2012). This decisive function is not reflected in high plasmodesmal densities, but most likely in the abundance of secondary plasmodesmata at the tangential non-division interfaces (Seagull 1983; Zhu et al. 1998). Similarly, to exert its function in stem cell maintenance in the SAM, the WUSCHEL transcription factor has to move symplasmically in apical direction across the periclinal non-division interfaces of the L1, L2, and L3 (Yadav et al. 2011; Fig. 2.1), where most plasmodesmata can be

expected to be secondary in origin. It is likely that the secondary plasmodesmata have special functional qualities for macromolecular transport. This idea has emerged earlier (for a recent review, see, e.g., Niehl and Heinlein 2011) and was critically discussed by Ehlers and Kollmann (2001) and Burch-Smith et al. (2011a), since the identification of "secondary plasmodesmata" was sometimes disputable (e.g., Ding et al. 1992b; Volk et al. 1996; Itaya et al. 1998).

2.6.4 Evolution of Plasmodesmata

In view of specialized functional tasks fulfilled by the secondary plasmodesmata in the plant tissues, it would be interesting to follow the evolution of these cell connections, but information is scarce. Although plasmodesmata are typical structures of all embryophytes (e.g., Cook and Graham 1999; Raven 1997, 2005), not all taxa seem to be able to form secondary plasmodesmata. Evidence for the formation of simple and unbranched secondary plasmodesmata in mosses comes from studies on developing *Sphagnum* leaflets (Schnepf and Sych 1983). Moreover, branched, and possibly secondary, plasmodesmata were found in the liverwort *Monoclea gottschei* (Cook et al. 1997). With ferns, however, no indications for secondary plasmodesmata formation were found in developing *Azolla* roots (Gunning 1978) and in gametophytes of *Onoclea sensibilis* (Tilney et al. 1990). These observations were recently supported by an extensive study on the plasmodesmal networks in the SAMs of twenty-four species of vascular plants (Imaichi and Hiratsuka 2007). The authors found a demarcation between fern-type SAMs which had at least threefold higher plasmodesmal densities at the interfaces of the meristematic cells than seed plant-type SAMs. The occurrence of two SAM types was attributed to the (dis)ability to form secondary cell connections (Imaichi and Hiratsuka 2007). Ferns, which are unable to generate secondary plasmodesmata, rely on the formation of high numbers of primary plasmodesmata. Remarkably, however, members of the lycopod families Isoetaceae and the Lycopodiaceae had SAMs of the seed plant-type, which may point to the capability for secondary plasmodesmata formation in these plant families (Imaichi and Hiratsuka 2007). Collectively, these findings may indicate that secondary plasmodesmata formation has evolved independently in mosses, Isoetaceae/Lycopodiaceae, and seed plants. Alternatively, the ability to form secondary plasmodesmata has evolved early in the evolution of the land plants, and was lost in monilophyte ferns, including Psilotum and Equisetum, and in the Selaginellaceae. Further investigations, including functional studies on the plasmodesmata in different plant taxa, might deliver further insights into the (special?) role of secondary plasmodesmata during plant development.

Investigations on plasmodesmata in green and brown algae are not restricted to the studies reviewed by Cook and Graham (1999) and Raven (1997, 2005). Fortunately, there are several recent studies on the cytokinesis and plasmodesmal formation in the Phaeophyta (Katsaros et al. 2009; Nagasato et al. 2010; Terauchi et al. 2012; and literature cited therein). In this taxon, cytokinesis is performed by

fusion processes of Golgi vesicles and flat cisternae in the cytokinetic plane to form membranous sacs which grow out and fuse to build the new cell partition membranes (Katsaros et al. 2009; Nagasato et al. 2010). ER-free, simple plasmodesmata develop from tubular membrane protrusions which are inserted into the membranous sacs (Katsaros et al. 2009; Terauchi et al. 2012). Although it has been discussed that the flat cisternae in the cytokinetic plane may be derived from the ER, since there was a close spatial association (Nagasato et al. 2010), the ER obviously has no significant role in the formation and in the final structure of the plasmodesmata in brown algae. In view of the central function attributed to the ER in the formation and structural modification of plant plasmodesmata, this finding is remarkable and needs further attention.

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Chapter 3
Regulation of Intercellular Transport Through Plasmodesmata Under Abiotic Stresses

Anna Bilska

Abstract Plants are often subjected to environmental stresses. Except many responses, unfavourable conditions may affect intercellular communication by significantly changing the rate and efficiency of symplasmic transport. This can involve changes in the ultrastructure of plasmodesmata. Furthermore, the modification can concern the surroundings of plasmodesmata through deposition of callose. Thus, understanding the mechanisms that control permeability of plasmodesmata under stress conditions is of fundamental importance. This chapter presents structural changes of plasmodesmata in relation to the symplasmic transport efficiency under abiotic stress conditions. Responses of plasmodesmata to external stimuli are discussed in terms of plant acclimation to unfavourable conditions. The role of plasmodesmata in plant adaptation to different habitats is also considered.

Keywords Abiotic stresses • Ecophysiology • Plasmodesmata ultrastructure • Sphincter • Symplasmic transport

Abbreviations

ER	Endoplasmic reticulum
PCA	Primary carbon assimilation
PCR	Primary carbon reduction
PD	Plasmodesma/plasmodesmata
SEL	Size exclusion limit
ROS	Reactive oxygen species

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3.1 Introduction

Under natural conditions, plants are often exposed to unfavourable environmental factors. The negative impact of such factors on the plants is commonly referred to as an abiotic or biotic stress. In general, any fluctuation in the habitat generates a defensive response of the plant, which can be manifested in different ways. The repertoire of the stress-induced changes depends on the type and the intensity of the stress. Furthermore, the stress response may be species or variety specific. Additionally, it can be expected that the magnitude of the changes will be higher when several stresses act simultaneously. It should also be noted that a long-term stress can lead to irreversible damage and consequently to plant death. On the other hand, a moderate stress can promote plant acclimation to suboptimal conditions allowing it to survive a subsequent severe stress.

Among the many aspects of the plant response to stress and, generally, of the plant ecophysiology, the role of plasmodesmata seems to be underestimated. Exogenous factors may strongly affect intercellular communication by significantly changing the rate and efficiency of symplasmic transport. This can involve changes in the ultrastructure of plasmodesmata, including the conformation of proteins (e.g. actin, myosin) associated with plasmodesmal microchannels or reorganisation of internal sphincters. Furthermore, the modification can concern the surroundings of plasmodesmata through deposition of polysaccharides (e.g. callose, pectins) in the plasmodesmata-enriched areas of the cell wall, so-called pit fields, and/or extracellular sphincters. Considering that plasmodesmata are "the main contractors" of symplasmic transport, understanding the mechanism that controls their permeability under stress conditions is of fundamental importance. This chapter presents structural changes of plasmodesmata in relation to the symplasmic transport efficiency under abiotic stress conditions. The influence of stress on the density, general shape and the proteinaceous and non-proteinaceous components of plasmodesmata are discussed. The nature and putative roles of inter- and extracellular sphincters/collars in the regulation of permeability of plasmodesmata are considered. Finally, the relationship between environmental factors, the plant reaction to stress involving plasmodesmata and the adaptation is discussed.

3.2 The Ultrastructure of Plasmodesmata

Plasmodesmata are cytoplasmic tubules up to about 50 nm in diameter (Zambryski and Crawford 2000; Roberts and Oparka 2003; Lucas and Lee 2004) composed of lipid-protein membranes and proteins. In higher plants, the centre of the plasmodesma is occupied by a desmotubule of a diameter of ca. 15–20 nm, an appressed form of the endoplasmic reticulum linking adjacent cells. The desmotubule is visible by electron microscopy as an electron-dense structure, also called the central rod (Hepler 1982; Ding et al. 1992; Botha et al. 1993; Overall and Blackman 1996;

Botha and Cross 2000; Ehlers and Kollmann 2001; Brecknock et al. 2011; Tilsner et al. 2011). According to Tilsner et al. (2011), the extreme constriction of the lipid bilayer forming the desmotubule is reached by protein–protein interactions. However, the nature of the proteins forming the scaffold of the desmotubule is unknown, and the authors (ibid.) suggest that they could be similar to the animal CLIMP-63 protein. The space between the desmotubule and the plasma membrane forms the so-called cytoplasmic sleeve. The desmotubule is surrounded by an annular helix composed of globular proteins and is linked by spoke-like structures to plasma membrane proteins (Robards 1968; Robinson-Beers and Evert 1991; Ding et al. 1992; Botha et al. 1993; Ehlers and Kollmann 2001). This ensures the mechanical/physical stability of plasmodesmata. Several authors (Overall and Blackman 1996; Baluška et al. 2001) have proposed that those spokes could consist of F-actin, myosin VIII and centrin. Myosin VIII may play a key role in the regulation of gating via plasmodesmata (Baluška et al. 2001). The spokes are separated from one another by microchannels of 3–4 nm (Lucas et al. 1993; Blackman and Overall 2001; Roberts and Oparka 2003). Plasmodesmata are often constricted at both ends forming neck regions, while their central regions are dilated. In some plasmodesmata, electron-dense structures called "sphincters" (Evert et al. 1977) have been reported. Consequently, numerous models of plasmodesmata with putative sphincter(s) were proposed (Ding et al. 1992; Overall and Blackman 1996; Roberts and Oparka 2003). In the model of Overall and Blackman (1996) the electron-dense elements form a helix surrounding the entire plasmodesma (Overall et al. 1982; Badelt et al. 1994), or ring-like structure around the neck region (Badelt et al. 1994). The latter structures are called extracellular sphincters (Olesen and Robards 1990; Lucas et al. 1993; Ritzenthaler et al. 2000; Rinne et al. 2001; 2005). Those authors proposed that these structures could consist of callose, which was later confirmed by immunolocalisation—antibodies against β-1,3-glucan visualised callose epitopes in the sphincter areas. However, sphincter-like structures may also occur inside plasmodesmata (Evert et al. 1977; Olesen 1979; Robinson-Beers and Evert 1991; Botha et al. 2005). In the case of such internal sphincters, the electron-dense particles fill the cytoplasmic sleeve at one or both neck regions. Despite the numerous cytochemical studies, the chemical composition of the internal sphincters is unclear. Using diverse chemicals, Turner et al. (1994) excluded the possibility of a proteinaceous character of the sphincters. However, electron microscopic observations of carefully fixed material showed that components of the electron-dense material present at the neck region of plasmodesmata have a globular shape (Olesen 1979; Overall et al. 1982; Robinson-Beers and Evert 1991; Botha et al. 1993), suggestive of proteins. Recently, electron tomography studies confirmed these observations for some types of leaf plasmodesmata in maize (Bilska and Sowiński 2010). 3D visualisations of electron tomography microphotograph series made for several types of plasmodesmata are shown in Fig. 3.1. Here, internal sphincters composed of clearly globular components are present at the both ends of plasmodesmata at the Kranz mesophyll/Kranz mesophyll interface and at the mesophyll side of the Kranz mesophyll/bundle sheath interface (Fig 3.1a, b) but not at other interfaces (Fig 3.1c). The proposed proteinaceous character of the internal sphincter components should

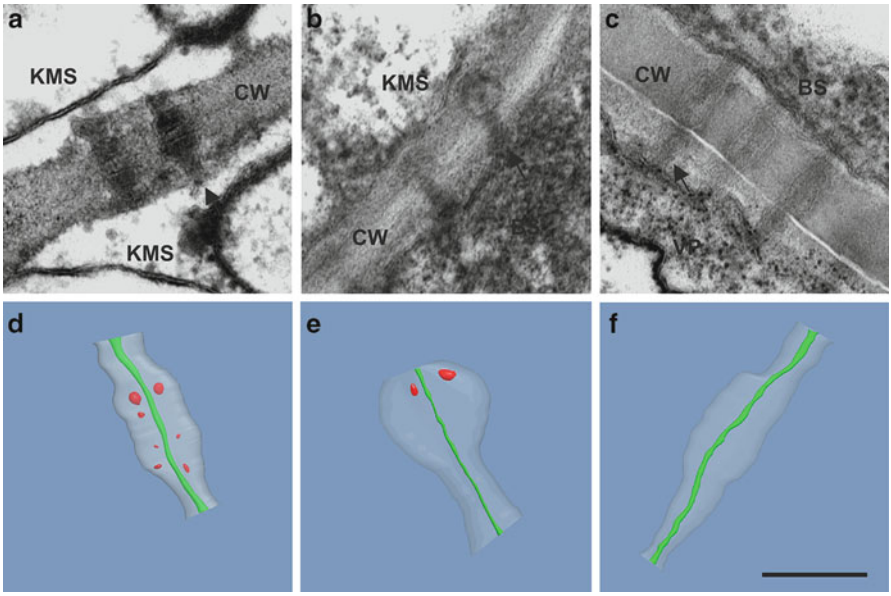

Fig. 3.1 Transmission electron micrographs (**a**, **b**, **c**) and a 3D visualisation (**d**, **e**, **f**) of three different types of plasmodesmata in maize leaf. (**a**, **d**) Leaves at the interface: Kranz mesophyll cell/Kranz mesophyll cell. (**b**, **e**) Kranz mesophyll cell/bundle sheath cell. (**c**, **f**) Bundle sheath cell/vascular parenchyma cell. Note globular elements at the plasmodesmata neck region in the two types of plasmodesmata (**a**, **b**, **d**, **e**). *KMS* Kranz mesophyll, *BS* bundle sheath, *VP* vascular parenchyma, *CW* cell wall. *Arrows*—PD which were taken in the model creations. Scale bar—200 nm (**a**, **b**, **c**), 100 nm (**d**, **e**, **f**). Three-dimensional shape of plasmodesmata was designed using 3D CAD software (Pro/ENGINEER Software, Wf2; Parametric Technology Corporation, USA) on the basis on electron tomography micrographs, performed by JEM 1400 (Jeol Co., Japan) equipped with a tilt-rotate tomographic holder and a high-resolution digital camera (CCD MORADA; Olympus Soft Imaging Solutions, Germany) in Laboratory of Electron Microscopy of Nencki Institute of Experimental Biology, PAS, Poland

be confirmed directly, using immunocytolocalisation and/or proteomics. In the latter case, the biggest problem for one studying protein composition of plasmodesmata is to obtain the material for studies (Faulkner and Maule 2011). It is also possible that soluble proteins or proteins transiently connected with plasmodesmata may be lost during the preparation of material (Salmon and Bayer 2013).

It should be emphasised that despite the great progress in the development of microscopy techniques, the ultrastructure of plasmodesmata is far from being fully established, mainly due to technical problems. Details of these minute structures are visible only under electron microscope. Standard electron microscopy requires multistep chemical preparation of the samples where, as a rule, material must be fixed immediately after the sample collection in order to several steps of sampling procedure: postfixation, dehydration, resin embedding, resin polymerisation and specimen cutting. This protocol may lead to structural artefacts, e.g. constriction of

plasmodesmata. Standard fixation can also damage sensitive epitopes and prevent immunocytolocalisation of proteins of interest dependent on proper recognition of the protein by a specific antibody.

Different modifications of classical fixation methods are known which improve the quality of fixation. Thus, the use of tannic acid during fixation of plant material improves visualisation of some structures under electron microscopy, including sphincters (Olesen 1979) and the striations linking the desmotubule to the plasmalemma (Tilney et al. 1991). Tannic acid/heavy metal (e.g. osmium) complexes form a stable film which protects native conformation of proteins and reduces damage dehydration. Non-chemical fixation techniques, such as low-temperature fixation (McIntosh et al. 2005), freeze substitution or freeze etching, are also useful in visualising substructures of plasmodesmata, including sphincters or collars (Northote and Lewis 1968; Willison 1976; Olesen 1979; Thomson and Platt-Aloia 1985; Badelt et al. 1994). Although damage to the freeze-substituted material is possible (Badelt et al. 1994) resulting in "stretching" of plasmodesmata, freeze-substitution methods are particularly well suited for the demonstration of plasmodesmata-associated material. The recent progress in confocal/fluorescence microscopy has allowed the study of structures below the level of light microscope resolution (Bell and Oparka 2011) and one should expect plasmodesma models based on *in vivo* observations soon.

3.3 Plasmodesmata and Plant Stress Responses

As for other physiological phenomena, the impact of a stress on symplasmic transport can be divided into the alarm and acclimation phases (Levitt 1980) comprising, respectively, a relatively rapid closure of individual plasmodesmata and a rather slow modification of cell-to-cell transport, due to changes in plasmodesmata frequency. The signal transduction pathways leading to those two types of responses are still under discussion.

3.3.1 Fast Modification of Cell-to-Cell Transport by External Stimuli

Numerous reports document rapid changes in symplasmic transport in response to external stimuli. The cell-to-cell transport of the fluorochrome Lucifer yellow was stopped immediately by an experimentally generated large (200 kPa) turgor pressure differential between leaf trichome cells of *Nicotiana clevelandii* (Oparka and Prior 1992), suggesting water potential sensing to be involved in the signal transduction. In another study, elevation of cytoplasmic free Ca^{2+} by cold treatment (2 °C) led to an increase of electrical resistance between cells in cell culture within

10 s, indicating a shift of the plasmodesmata from an open to a closed conformation (Holdaway-Clarke et al. 2000) and also showing the participation of this secondary messenger in the transduction of the external signal. Recently, reactive oxygen species (ROS) have been shown to affect the modulation of plasmodesmata state (Benitez-Alfonso and Jackson 2009; Benitez-Alfonso et al. 2009; Stonebloom et al. 2009; Benitez-Alfonso et al. 2011; Stonebloom et al. 2012). Alteration of the redox state in mitochondria increased the transport via plasmodesmata (Stonebloom et al. 2009) while plastid-produced ROS blocked symplasmic transport (Benitez-Alfonso and Jackson 2009; Benitez-Alfonso et al. 2009). It seems, therefore, that several different signal transduction pathways could control the symplasmic transport. One should note, however, that different signal transduction pathways may converge (Fryer et al. 2003; Cessna et al. 2007) to produce cross-tolerance (Bowler and Fluhr 2000).

Several lines of evidence have confirmed the participation of all three signal transduction pathways mentioned above in the regulation of plasmodesmata opening. The findings of Schulz (1995), who demonstrated relatively fast (1 h) opening of PD in roots of *Pisum sativum* in response to osmotic stress (accompanied by increased phloem unloading), support the concept of the water potential of the cell being a regulator of symplasmic transport. On the other hand, plasmolysis of cortical cells using 1 M sucrose reduced both the number of plasmodesmata classified as "open" and the phloem unloading. The cytoplasmic free calcium hypothesis seems to be confirmed by a study in which plasmodesmata in the staminal hairs of *Setcreasea purpurea* closed as a result of the elevation of intracellular calcium (Tucker 1990; Tucker and Boss 1996). In other experiments, the effect of light on the symplasmic transport in etiolated corn seedlings was determined (Epel and Erlanger 1991). Those authors suggested an involvement of phytochrome in this control. The phytochrome-mediated photomorphogenetic responses require, among other factors, calcium (Neuhaus et al. 1997; Frohnmeyer et al. 1998). Also the postulated role of redox signalling in the control of plasmodesmata closure seems to have strong experimental support. One such line of evidence comes from studies of a maize *sxd1* mutant showing tocopherol deficiency (Sattler et al. 2003). The mutant's phenotype involves closure of plasmodesmata at the bundle sheath/vascular parenchyma interface (Russin et al. 1996). It has been suggested recently that ROS accumulation due to the tocopherol deficiency could be the factor responsible for the plasmodesmata closure (Benitez-Alfonso et al. 2011). Also, in line with the ROS hypothesis is our finding that moderate chilling (14 °C) slows down the short-distance transport of ^{14}C-photosynthates and phloem loading in *Zea mays indentata* leaves (Bilska and Sowiński 2010) and is accompanied by closure of plasmodesmata at the Kranz mesophyll/bundle sheath and bundle sheath/vascular parenchyma interfaces, i.e. those involved in the exchange of C4 photosynthates between the PCA and PCR cycles and in the symplasmic transport of sucrose prior to phloem loading. Moderate chilling causes photoinhibition in *Zea mays indentata* (ibid.), but not elevation of free cytoplasmic calcium (Bilska and Sowiński, unpublished data); thus, the observed closure of plasmodesmata could well be due to enhanced ROS production, but not to calcium signalling.

3.3.2 *Mechanisms of Rapid Response of Plasmodesmata to External Stimuli*

The most frequently discussed aspect of the ultrastructural changes of plasmodesmata under stress conditions is accumulation of callose at the neck regions, sometimes forming extracellular sphincters. One should note, however, that the changes in symplasmic transport demonstrated by some to occur within seconds (Oparka and Prior 1992; Holdaway-Clarke et al. 2000) are much too rapid to be due to callose deposition, taking several minutes at least (Chen and Kim 2009; Zavaliev et al. 2011). It seems likely that different mechanisms are responsible for the fast and delayed closure of plasmodesmata.

3.3.2.1 Plasmodesmata Constriction Related to Callose

Callose (β-1,3-glucan) plays several roles in higher plants including development of pollen, cytokinesis (Chen and Kim 2009) and blockage of sieve pores in trees at the end of vegetation season (dormancy callose). The polysaccharide also deposits at the neck regions of plasmodesmata in response to diverse stresses. Its function has been discussed in the literature mostly in the context of biotic stresses and transport of macromolecules (see Chap. 7). However, callose is also deposited at plasmodesmata following of abiotic stresses (Table 3.1). Thus, plasmodesmata of wounded roots of onion (*Allium cepa* L.) demonstrated stronger constriction of the neck region than in control, unwounded tissues (Radford et al. 1998), which was apparently a result of callose accumulation: when an inhibitor of callose synthesis, 2-deoxy-D-glucose (DDG), was used, no neck constrictions were observed in the plasmodesmata. Several reports also showed callose accumulation at plasmodesmata following a metal stress: aluminium (Sivaguru et al. 2000), lead (Piršelová et al. 2012; Samardakiewicz et al. 2012), arsenic (Piršelová et al. 2012) or cadmium (Ueki and Citovsky 2005; Piršelová et al. 2012). In the case of cadmium and arsenic acting on soybean roots (Piršelová et al. 2012), callose deposits were found in the epidermis, cortex and endodermis. The authors suggested that the deposits of callose at plasmodesmata in the epidermis and cortex are elements of the defence against penetration by the toxic metal (ibid.). However, a study on root cells of *Lemna minor* L. by Samardakiewicz et al. (2012) indicated that the effectiveness of callose as a barrier preventing of toxic metal ion translocation depends on the distribution of this polysaccharide in the cell.

Callose may plug plasmodesmata by either forming a collar at the neck region or along the whole plasmodesma, thereby reducing its diameter (Olesen and Robards 1990, Radford et al. 1998). Callose is synthesised from UDP-glucose at the site of its deposition by callose synthase (EC 2.4.1.34) and degraded by hydrolysis catalysed by β-1,3-glucanase (EC 3.2.1.39). Levy et al. (2007) demonstrated that wounding stress applied to *Arabidopsis* mutants that lack β-1,3-glucanase (AtBG_ppap-deficient mutants) led to a stronger accumulation of callose in comparison to

Table 3.1 Effect of abiotic stresses on the deposition of callose at plasmodesmata

Type of stress	Species/tissue	Localisation	Method of callose localisation	Method of transport observation	References
Wounding	Arabidopsis mutants/leaves	Epidermal cells	Aniline blue	GFP	Levy et al. (2007)
	Allium cepa L./ roots	Cortical cells	Aniline blue	Indirectly by PD ultrastructure analysis	Radford et al. (1998)
Osmotic stress	*Avena sativa* L./coleop-tile	Parenchyma cells	Aniline blue	Electrical coupling	Drake et al. (1978)
Metal stresses:					
Aluminium	*Triticum aestivum*/ roots	Root apex	Aniline blue; immuno-gold	LYCH	Sivaguru et al. (2000)
Arsenic	Soybean/roots	Epidermis, cortex, endodermis	Aniline blue	ND	Piršelová et al. (2012)
Cadmium	Soybean/roots	Epidermis, cortex, endodermis	Aniline blue	ND	Piršelová et al. (2012)
	Maize/roots	Central cylinder, epidermis, cortex	Aniline blue	ND	Piršelová et al. (2012)
Lead	*Lemna minor* L./roots	Protoderm, procambial central cylinder	Aniline blue; immuno-gold	ND	Samardakiewicz et al. (2012)
Cold	*Zea mays* L./leaves	Cell walls in the vascular tissues	Immunogold technique	Isotope technique	Bilska and Sowiński (2010)

PD plasmodesmata, *GFP* green fluorescent protein, *LYCH* Lucifer yellow carbohydrazide, *ND* not determined

wild-type plants. Those authors discussed the roles of the β-1,3-glucanase and β-1,3-glucan synthase activities in controlling the permeability of plasmodesmata via callose breakdown and accumulation.

Several lines of evidence have shown that stress-induced accumulation of callose at plasmodesmata leads to the blockage of symplasmic transport (Rinne and van der Schoot 1998; Sivaguru et al. 2000; Radford and White 2001; Rinne et al. 2001; Levy et al. 2007) or participates in stress signal transduction (Vatén et al. 2011). Using confocal microscopy and green fluorescent protein (GFP), Levy et al. (2007) showed that after wounding, the dye diffusion between epidermal cells of leaves of mutants expressing high level of plasmodesmata-associated callose is slower in the comparison to the wild type. The microinjection of a fluorescent dye (LYCH) into root cells of aluminium-stressed wheat also indicated an inhibition of cell-to-cell

communication (Sivaguru et al. 2000). Those authors postulated that callose effectively limits symplasmic transport in response to the metal stress.

The mechanism of plasmodesmata gating by callose has been discussed mostly in the context of a pathogen attack (Zavaliev et al. 2011), while the signal transduction pathway linking abiotic stresses and callose deposition at PD is unknown. An interesting hypothesis addressing that question has been formulated recently by Benitez-Alfonso et al. (2011). They propose that the enhanced ROS production induced by different biotic and abiotic factors may increase the high cytosolic Ca^{2+} concentration by opening Ca^{2+} channels of tonoplast and membranes of other Ca^{2+} reservoirs thereby induce the expression of genes encoding plasmodesmata-related proteins. This would lead to activation of callose synthase and modification of the plasmodesmata ultrastructure and size exclusion limit (SEL).

3.3.2.2 Plasmodesmata Constriction Not Related to Callose Deposition

A rapid response of plasmodesmata to external stimuli can be generated by changes in the organisation of calcium-dependent proteins—centrin and calreticulin. Blackman et al. (1999) proposed two models to explain the role of centrin in the regulation of transport via plasmodesmata. In the first model, centrin nanofilaments link the endoplasmic reticulum to the plasma membrane. When the intracellular calcium level increases, the centrin filaments rapidly contract, thereby pulling the ER against the plasmalemma. In the second model, centrin traverses the plasmodesma channel in the neck region and connects to the plasma membrane. In response to a rising of free cytoplasmic calcium concentration, the constricting centrin filaments pull the plasma membrane inwards. In this case the plasmodesma can be completely sealed by overpackaging of centrin filaments in the neck region. Another protein involved in stress-induced changes of plasmodesmata permeability is myosin. Wojtaszek et al. (2005) demonstrated the relocation of plant-specific myosin VIII to pit fields in root cap protoplasts exposed to the osmotic stress (90 min) which induced their plasmolysis. They suggested that the relocation of myosin VIII is accompanied by rearrangement of actin filaments that both those changes are part of an actomyosin mechanism of osmosensing in the root.

Apart from the contraction of plasmodesmata, another mechanism that should result in the cessation of symplasmic transport could involve swelling of internal sphincter. In line with the opinion of several authors that the ring-shape internal sphincters at the neck region of plasmodesmata could control the symplasmic transport (Evert et al. 1977; Anisimov and Egorov 2002; Botha et al. 2005) was our observation of swelling of elements of internal sphincters in an inbred line of *Zea mays indentata* in parallel with the slowing down of the rate of transfer of ^{14}C-photosynthates to the phloem after a short treatment (1 h) with low temperature (Bilska and Sowiński 2010). One should note that the cold-induced swelling of the sphincter elements was accompanied by accumulation of calreticulin at the neck region of the plasmodesmata (ibid.). The participation of calreticulin, a calcium-sequestering protein, in the control of SEL was also postulated by other authors

(Baluška et al. 1999; Blackman et al. 1999). Unfortunately, no other proteins could be immunolocalised at the neck regions of the maize leaf plasmodesmata showing cold-induced sphincter enlargement. In particular, despite trying various antibodies, fixation and immunolocalisation procedures, we failed to detect centrin (Bilska et al. unpublished data), whose role in the control of SEL was discussed above. Apparently, either centrin is not a component of maize plasmodesmata or its maize variant could not be recognised by the antibodies used. The nature of the ring-shaped sphincter and mechanism of its enlargement are unknown. Since internal sphincters are present in numerous C4 grass species (Botha et al. 2005; Sowiński et al. 2007), including some of high economic importance, these structures and their role in the regulation of the flow of C4 photosynthesis intermediates deserve more interest in the future.

3.3.3 Slow Modification of Cell-to-Cell Transport in Response to External Stimuli

A clear-cut distinction between the alarm and acclimation phases of a stress is rarely possible, and this is also true for the plasmodesmata response to stress. A good example is the toxic metal stress-induced accumulation of callose at plasmodesmata. While callose accumulation at plasmodesmata can often be detected within minutes of the stress application suggesting the alarm phase of stress response, it apparently also increases the tolerance to the stress—conforming to the definition of acclimation response. Additionally, the data concerning the delayed response to stress in regards to PD are scarce. Hence, how the changes in symplasmic transport are related to other phenomena is mostly speculative. An example here are dehydrins, involved in acclimation to various environmental factors affecting water conditions, such as drought, salinity and low temperature in plant cells (Close 1997; Lee et al. 2005; Rorat 2006; Kosová et al. 2007). These proteins have also been localised at plasmodesmata in the vascular cambium cells of cold-acclimated *Cornus sericea* (Karlson et al. 2003). The authors considered the possible role of the plasmodesmata-associated dehydrin-like proteins—an indirect one in the structural protection of plasmodesmata, or direct, by minimising damage to the membranes due to freeze-induced desiccation.

Some indication of processes or genes likely involved in plant acclimation to abiotic stress and also related to plasmodesmata functioning comes from data mining of the results of numerous genome-wide transcriptomic experiments focusing on the plant response to stress. These data are always deposited in public databases, so they can be relatively easily analysed by anyone interested in the problem. Just to illustrate the point, by analysing data from a microarray experiment on cold-treated maize during the acclimation phase of stress (Trzcinska-Danielewicz et al. 2009), one comes across profound changes in the expression of several genes encoding proteins potentially related to PD, e.g. remorins, PT1 interactor and

TMV-MP30 binding protein (Herrmann et al. 2006; Wright et al. 2007; Raffaele et al. 2009). A search for databases of other microarray experiments mined several other known genes encoding PD-related proteins whose expression changed under stress indicates their potential participation in acclimation. Interestingly, the most of these genes are known as related to virus trafficking through plasmodesmata. Either signal transduction pathways for biotic and abiotic stresses cross each other or the roles that proteins discovered in studies on biotic stress are not specific for this type of stress.

As regards symplasmic transport, a long-lasting acclimation of a plant to a changing environment could be attained by the emergence of secondary plasmodesmata or changes in their type by modification of the pre-existing ones, e.g. by branching. Changes in plasmodesmata frequency at some developmental stages in response to endogenous cues are a very well-known phenomenon (see Chap. 2), but, unfortunately, the accommodation of the symplasmic network to environmental requirements has not been studied thoroughly. In one of only few reports in this field, Sowiński et al. (2003) reported that the frequency of plasmodesmata (in particular those at the Kranz mesophyll/bundle sheath and bundle sheath/vascular parenchyma interfaces, responsible for exchange of C4 intermediates and prephloem transport of photosynthates, respectively) in leaves of *Zea mays indurata* grown at a low temperature was significantly higher than in control. That was accompanied by some acclimation of the photosynthetic apparatus to the chilling. Another example of the symplasmic network acclimation came from studies of plant adaptability to growth light conditions. Amiard et al. (2005) demonstrated that the frequency of plasmodesmata in leaves of plants with the symplasmic phloem loading mode (see Chap. 5) was the same for plants grown at low light intensity (LL), high light (HL) intensity or transferred from low light to high light. On the other hand, plants grown at a high light intensity showed a higher density of veins than in the other two experimental variants. Hence, overall, the symplasmic network was denser in HL plants than in either LL plants or those transferred from LL to HL. Another example of the symplasmic network adaptability to growth light conditions was reported for several species of C4 grasses (Sowiński et al. 2007). These plants showed higher plasmodesmata frequency in leaves (in particular plasmodesmata at Kranz mesophyll/bundle sheath interface) when grown at higher growth light intensities. In parallel, also the photosynthetic efficiency of the leaves was higher. The signal transduction pathway linking the growth light conditions with the symplasmic network is unknown. The link between the symplasmic network density and the growth light conditions resembles the well-known phenomenon that sinks often pre-adapt the vasculature size to the potential demand for assimilates (Morris 1996 and literature cited there). Since both adaptive phenomena take place at early stages of development, one may hypothesise that they represent photomorphogenetic responses. Further studies are necessary to asses if the change of symplasmic network density as a mechanism of acclimation to environment fluctuation is not limited to few examples described in the literature.

3.4 Plasmodesmata and Plant Adaptations to Different Habitats

Adaptation is one of the mechanisms allowing plants to resist environmental stresses. It is defined as heritable modifications of organisms of a given taxon that allow them to survive under given environmental conditions (Levitt 1980). Adaptation clearly concerns also the cell-to-cell transport, underlining the importance of the symplasmic network for plant ecophysiology, i.e. the mutual relations of plants and their environment.

The ability of many plants to overwinter is an elegant example of adaptation to the temperate zone. It is related to frost hardening in the autumn that allows the plant to survive often severe freezing temperatures during winter. As shown by Rinne et al. (2001), for birch (*Betula pubescens*), short days lead to uncoupling of cell-to-cell contacts and stop the symplasmic transport in apical meristems, thus causing bud dormancy. This is related to accumulation of callose in external sphincters. The breaking down of the bud dormancy requires a combination of endogenous processes, involving restoration of symplasmic coupling in the apical meristem by β-1,3-glucanase (breaking down the endodormancy), and exogenous factors such as water availability and temperature rise (breaking down the ecodormancy). These processes together form a synergic mechanism that allows the plant to anticipate the seasonal environment fluctuations (thanks to it, plant can enter the dormancy before a real risk from winter temperatures), to stay dormant during the winter despite short-term temperature fluctuations and to wake up at spring.

Another example of a cyclic endogenous process that allows the plant to fit well in the environment and depends on modification of the cell-to-cell transport, albeit at a different time scale, is circumnutation (Brown 1993; Stolarz 2009). It is a cyclic autonomous helical organ movement of diverse character depending on the plant species, of a period of several minutes to several hours. Circumnutation allows climbing plants to seek for solid support for growth, and other roles have been postulated as well (Stolarz 2009). Among the models of the mechanism of circumnutation, one based on changes in cell-to-cell transport seems the least controversial. The model proposed by Brown (1993) posits that circumnutations are powered by an asymmetric development of plasmodesmata in growing regions, leading to uneven symplasmic transport of growth substances and consequent nutations. The model assumes cyclic functional disruption and restoration of plasmodesmata. To follow changes in the symplasmic transport in circumnutating organs and the mechanism of the modulation of the plasmodesmata conductivity would be challenging subjects to study for those interested in the cell-to-cell communication. Such a study awaits to be performed and its impact could be substantial considering that climbing plants are of major economic importance to mention the grapevine, numerous bean species, the kiwifruit and many others.

Last but not least, a crucial adaptation related to symplasmic transport in the context of plant ecophysiology is the adjustment of the vein ultrastructure to diverse phloem loading mechanisms in higher plants. The different modes of phloem

loading are discussed thoroughly in Chap. 5. Here, only some aspects related to ecophysiology are addressed. The symplasmic mode of phloem loading related to open vein ultrastructure characterised by high frequency of plasmodesmata linking companion cells with the adjoining parenchymatic cells (type 1, 1-2a) was assumed by Gamalei (1991) to be specific for the tropical climate. The author hypothesised (Gamalei 1989) that mode of phloem loading was more primitive than the apoplasmic one related to a closed vein ultrastructure characterised by symplasmic isolation of companion cell/sieve element complex (type 2b). Further studies have refuted the simple idea of the evolution from open to closed veins and from symplasmic mode of phloem loading to the apoplasmic one. A recent report by Davidson et al. (2011) has demonstrated that symplasmic phloem loading could rather be associated with the tree growth form, while apoplasmic with the herbaceous habit. It is also worth noting that among species with an open vein structure, many are isoprenoid emitters (Kerstiens and Possell 2001). Isoprenoids (terpenes) are a group of volatile substances emitted by many plants that play diverse roles, among them improving the tolerance of high temperatures (Sharkey and Yeh 2001). Isoprenoid synthesis is promoted by high carbohydrate levels in the leaf, so one could expect higher isoprenoid emission in plants with the symplasmic phloem loading mode (Logan et al. 2000). Thus, the open vein ultrastructure and symplasmic phloem loading seem to be a fine example of an adaptive trait allowing tropical plants to thrive in hot environments. Another one is the vein structure type 2c, specific to C4 plants (described in more detail in Chap. 1). These plants have few plasmodesmata between the companion cell/sieve element complex and adjoining cells but numerous plasmodesmata between bundle sheath cells and mesophyll cells. C4 plants are best adapted to open, dry and hot habitats (Pearcy and Ehleringer 1984; Sage and Pearcy 2000), and so is their symplasmic network.

3.5 Conclusions

Biotic and abiotic stresses affect profoundly the plant growth and development and are the source of major environmental constrains to agriculture. A vast majority of studies related to cell-to-cell communication in the context of stress response concern virus and pathogen spreading in plants. In contrast, the mechanisms of the plant response to abiotic stresses related to plasmodesmata functioning and symplasmic network dynamics have only been addressed in a few scattered reports. It is therefore difficult to assess whether the modifications of the cell-to-cell communication in reaction to diverse stress types have the same or different molecular bases. The evolutionary adaptations of plants to different habitats by symplasmic network modifications are even less known, which seems unexplainable in view of their fundamental importance for the agriculture.

Several problems seem to be particularly pressing here, such as the rapidly increasing area and extent of soil pollution by salinity or toxic metals and other chemicals. In that context, the role of the symplasmic component in transport and

exclusion of excess salt and toxic substances deserves more attention, since the transport of solutes in roots and to the salt glands and trichomes on leaves is, at least partially, symplasmic. The recent discovery of a possible control over SEL by redox signalling poses the question on the timescale of the plasmodesma response to a redox balance change. If the response turns out to be fast, it could constitute a novel mechanism of modulation of processes dependent on symplasmic transport, e.g. C4 photosynthesis, cell-to-cell transport of solutes in symplasmic loaders or phloem unloading. From the ecophysiological point of view, the spontaneous or selection-assisted adaptation of plants involving modification of plasmodesmata frequency to habitats changed by human activity opens a new area for research and applied studies. One should expect an increased symplasmic network density under elevated carbon dioxide conditions, similarly as in high growth light, at least in symplasmic phloem loaders. Such an adaptation would allow plants to function well in the changing climate.

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Chapter 4
Symplasmic Transport in Wood: The Importance of Living Xylem Cells

Katarzyna Sokołowska

Abstract Short- and long-distance transport in woody plants is performed within two different systems of apoplasm and symplasm. Although the apoplasmic route of transport via dead conductive elements dominates in trees, the presence of interconnected living cells in xylem suggests the involvement of the symplasmic route in the transportation and communication processes occurring in wood. In this chapter, an attempt will be made to demonstrate and review the numerous functions of living xylem cells and the role of symplasmic transport in the secondary xylem of seed plants. The anatomical and ultrastructural characteristics of xylem parenchyma, reflecting the participation of living cells in the accumulation and distribution of stored compounds via symplasmic routes, will be presented. The involvement of living xylem parenchyma cells in the aspects of sugar transport and exchange at the symplasm/apoplasm interface, embolism repair, defense mechanisms against vascular pathogenic infection, and differentiation processes of xylem elements will be reviewed to emphasize the crucial impact of symplasmic transport and communication processes, via plasmodesmata, on integration and proper functioning of trees.

Keywords Axial parenchyma • Contact cells • Embolism repair • Isolation cells • Sapwood • Secondary xylem • Storage parenchyma • Sugar influx • Sugar efflux • Symplasm/apoplasm interface • Xylem rays

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4.1 Introduction

Secondary xylem is a heterogeneous tissue composed of both dead and living cells. Conductive elements like tracheids and vessels, as well as strengthening dead fibers, form the apoplasmic system, which itself is responsible for the long-distance transport of water and solutes in woody plants. In addition, a counterpart system for short- and long-distance symplasmic transport is formed by the living fibers and parenchymatous cells of the secondary xylem. Both the apoplasmic and symplasmic systems are closely integrated and cannot function alone. Thus, transport in woody plants has to be considered as a whole, as a union of apoplasmic and symplasmic parts. Fast apoplasmic transport prevails in the secondary xylem and thus is studied intensively (Holbrook and Zwieniecki 2005). However, the presence of a spatial and continuous system of different types of living cells in the secondary xylem region enables symplasmic communication in this tissue and provides efficient routes for short- and long-distance symplasmic transport in woody plants. Living xylem cells are involved in different physiological and developmental processes, where more careful control over intercellular transport and communication is necessary (Barnett 2006). For example, they provide routes for the transport of photoassimilate and solutes between living cells of the xylem, phloem, and cambial regions and between living and dead cells at the symplasm/apoplasm interface (Sauter 1982; Sauter and Kloth 1986; Améglio et al. 2004). They produce chemical weapons during vascular pathogen infection (Benhamou 1995), influence the differentiation processes of xylem elements (Lachaud and Mauroussset 1996), and participate in the refilling of embolized vessels (Salleo et al. 2004; Améglio et al. 2004). Although many of the processes involving living xylem cells are rather still unknown, we are slowly beginning to understand the significance of symplasm in the regulation and integration of woody plant function.

4.2 Living Cells Are Essential Components of Wood of Seed Plants

All the elements of secondary conductive tissues are produced by cambium, the lateral meristem of woody plants. Cambium is composed of two different types of initials—ray and fusiform—whose division activity gives rise to the radial and axial system of the secondary xylem and phloem elements. Thus, the living cells in wood, as derivatives of cambial cells, are incorporated into both of these two systems of radial and axial secondary conductive tissues (Larson 1994).

Additionally, two different regions can be distinguished within the volume of the secondary xylem. The outer wood region in the vicinity of cambium, where living xylem cells occur, is called sapwood. The inner wood region, close to the center of the trunk, which does not contain living cells and in which reserve storage materials are removed or converted into secondary metabolites, is called heartwood (Carquist 1988; Romberger et al. 1993).

4.2.1 The Living Cells of the Radial System

The radial system in wood is composed of rays—groups of cells that extend radially from the cambium in two directions: to the pith (centripetally) forming xylem rays and towards the cortex (centrifugally) creating phloem rays. Xylem rays are usually formed by living parenchymatous cells, although different specialized cells can additionally occur in some taxa of gymno- and angiosperms. For instance, rays can include ray tracheids (Bannan 1934, 1941), radial vessels (Botosso and Gomes 1982), radial fibers (Lev-Yadun 1994), as well as radially orientated canals or cavities filled with resin, mucilage, and oils (Carquist 1988; IAWA 1989; LaPasha and Wheeler 1990).

Rays can be classified as uniseriate, when they are only one cell wide, or multiseriate, where two or more cells are present in the widest point of the ray viewed at the tangential plane (Fig. 4.1). Another feature describing the rays from an anatomical point of view is their height, which is defined by the number of cells located between both (apical and bottom) ends of a particular ray along vertical line (Fig. 4.1; Carquist 1988; IAWA 1989). The living parenchymatous ray cells of angiosperm can be also subdivided into three different morphological types based on the shape of ray cells at the radial plane: upright when the cells are axially higher than wide, procumbent when the radial dimension of the cell is longer than its height, and square when cells are as tall as wide. If the ray is composed of only one cell type, e.g., exclusively procumbent, it is a homogenous ray. If other morphological types of parenchymatous ray cells or additional specialized elements, such as ray tracheids, are present, the ray is classified as heterogeneous (Fig. 4.1; Metcalfe and Chalk 1983; Carquist 1988; IAWA 1989). Finally, the xylem ray cells neighboring the tracheary elements can be divided into two categories (Fig. 4.2): contact cells which commonly have pits with tracheary elements and isolation cells that lack pit connections with tracheary elements or have sparse pitting (Höll 1975; Czaninski 1977; Gregory 1978; Murakami et al. 1999).

All features of ray anatomy, useful for taxonomists, are a direct consequence of the defined roles that particular xylem elements perform in the plant. In this light, the spacing and dimension of xylem rays result from the necessity to transport solutes in radial direction in woody plants. Rays are specialized highways for radial transportation within the xylem region and with neighboring tissues, especially towards to the cambium and phloem. Thus, their distribution has to be precisely regulated by the controlled spacing of ray initials in cambium. The latter is controlled by interactions between regulatory factors, presumably ethylene and auxin (Lev-Yadun and Aloni 1991, 1992, 1995). Formation of new ray initials and the enlargement of existing ones are induced by ethylene, while the presence of ray tracheids, vessels, and fibers within the rays results from radial auxin movement. Similarly the formation of specific morphological types of ray cells is the effect of hormonal interaction between axially transported auxin and radially spreading ethylene. If one of the hormones prevails, the cell shape changes in accordance with the direction of dominant hormone movement. If both of these hormones are balanced, the cells maintain a square shape (Lev-Yadun and Aloni 1991, 1995).

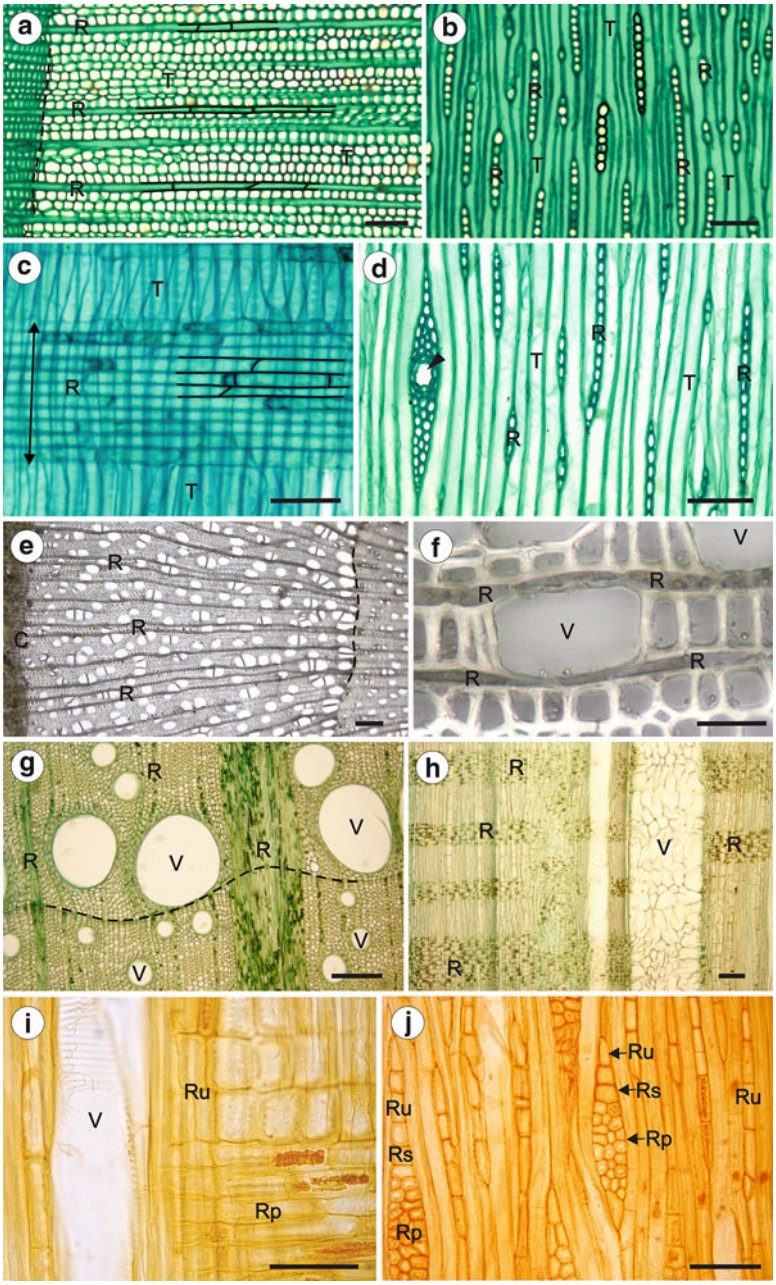

Fig. 4.1 The radial system of xylem parenchyma in seed plants. (**a**–**d**) Different types of xylem rays and their arrangement in the wood of gymnosperms. (**a**–**c**) Transverse (**a**), tangential (**b**), and radial (**c**) sections of *Abies* sp. wood. Homogenous rays, one cell wide (uniseriate), are frequent. The cell shape of some ray cells is outlined by *solid lines*; a double-head *solid line* indicates the height of the xylem ray. (**d**) Tangential section of *Picea* sp. wood. A heterogeneous multiseriate

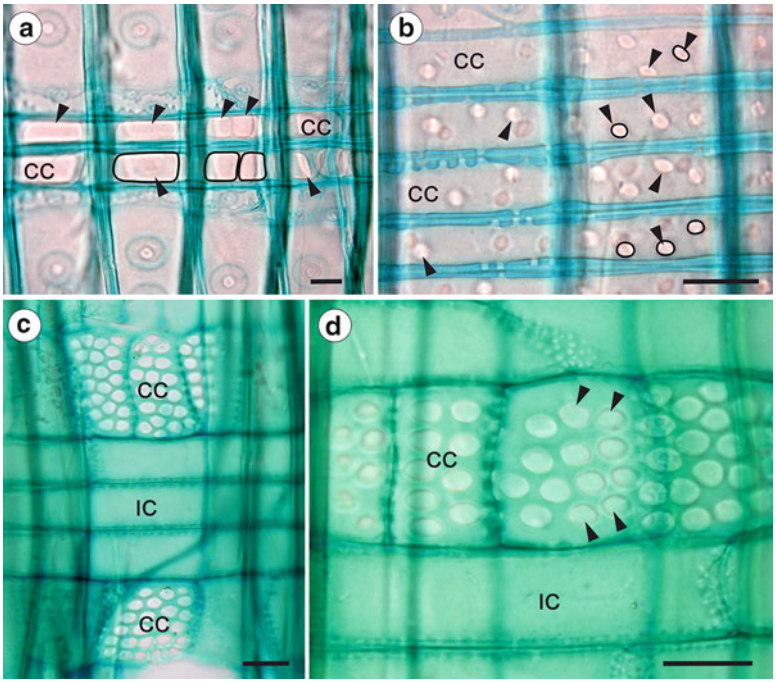

Fig. 4.2 Contact ray cells in seed plants. Xylem rays neighboring tracheids (**a**, **b**) and vessels (**c**, **d**) are presented in radial sections of the secondary xylem of *Pinus* sp. (**a**), *Picea* sp. (**b**), and *Salix* sp. (**c**, **d**). Large pits of contact cells are clearly visible; they are indicated by *arrowheads*; the borders of some contact pits are marked by *solid lines*. In heterogeneous rays of *Salix* sp., contact ray cells and isolation ray cells are labeled. *CC* contact cells, *IC* isolation cells. Microphotographs were taken in white transmitted light, scale bars: 20 μm (**a**, **b** courtesy of Dr. E. Myśkow)

Fig. 4.1 (continued) ray, containing a resin canal (indicated by an *arrowhead*), is visible between homogeneous uniseriate xylem rays. (**e**–**j**) Different types of xylem rays and their arrangement in the wood of angiosperms. (**e**) Diffuse-porous wood, containing multiple vessels with approximately similar dimensions, is shown in a transverse section of *Populus* sp. Frequent narrow xylem rays are present. (**f**) Magnification of a microphotograph (**e**). Uniseriate xylem rays surrounding vessel element are clearly visible. (**g**) The ring-porous wood, with large vessels produced at the beginning of the season and narrow vessels formed in its second part, is shown in a transverse section of *Quercus* sp. Frequent uniseriate and characteristic multiseriate rays are presented. (**h**) Horizontal bands of xylem rays are visible on a radial section of *Quercus* sp. wood. (**i**, **j**) Heterogeneous, multiseriate rays of *Cornus* sp. are shown in radial (**i**) and tangential (**j**) sections of the secondary xylem. Procumbent ray cells are present in the center of xylem rays, upright ray cells are visible at the ray margins, while square ray cells are located between procumbent and upright cells. *R* xylem ray, *Rp* procumbent xylem ray, *Rs* square xylem ray, *Ru* upright xylem ray, *T*, tracheid/s, *V* vessel; *dashed lines* show the border of annual ring. Microphotographs were taken in white transmitted light; scale bars: 100 μm (**a**–**e**, **g**–**j**) and 20 μm (**f**) (**g**–**j** courtesy of Dr. E. Myśkow)

The size of the rays changes during their ontogeny and strongly depends on plant growth rate. Rays become bigger as they age reflecting developmental events occurring in the cambial ray initials (Barghoorn 1941a; Evert 1961; Cumbie 1967; Ghouse and Yunus 1973; Myśkow and Zagórska-Marek 2004, 2008, 2013). The number of xylem rays and their volume in the total mass of the xylem region differ among vascular plants, but usually ranges between 8 and 25 % (Ghouse and Yunus 1974; Šćukanec and Petrić 1977; Gregory 1977), although in some tropical trees, e.g., *Dillenia indica*, the volume of ray initials can reach up to 75 % of the total volume of the wood (Ghouse and Yunus 1974). Moreover, in fast-growing trees, where radial growth leads to intensive circumference expansion, the ray size and the number of cells in particular rays increase rapidly (Bannan 1965; Gregory 1977). There is also a positive relationship between leaf area and the volume of ray parenchyma cells in outer sapwood (Gartner et al. 2000).

Rays may also affect cambial activity. One exquisite and striking example of this is the impact of rays on the maintenance of the meristematic character of cambial fusiform initials. The fusiform initials that are in direct contact with ray initials, or are in their close vicinity, maintain a meristematic identity. Conversely, the lack of such contacts and their more distant localization may induce the conversion of a fusiform initial into a new ray initial, or the loss of its meristematic character, leading to cell differentiation (Bannan 1965). The nature of signals transported from ray to fusiform initials, securing their meristematic state, is unknown.

4.2.2 Living Cells of the Axial System

Long-living cells of nucleated fibers and axial parenchyma cells can occur in the axial system of sapwood derived from cambial fusiform initials (Metcalfe and Chalk 1983; Carquist 2010). These cells are surrounded by secondary cell walls with numerous pits and can have a transverse septate. Living fibers and axial parenchyma have similar functions in woody plants. They are involved in sugar exchange at the symplasm/apoplasm interface (Sauter 1982; Améglio et al. 2004) and in the process of starch accumulation (Sinnott 1918; Ziegler 1964; Yamada et al. 2011). They also participate in long-distance symplasmic transport, through the formation of vertically orientated pathways for the spread of solutes, being simultaneously in direct contact with oppositely orientated rays (Fig. 4.3; van der Schoot and van Bel 1989; Sokołowska and Zagórska-Marek 2012).

Specific distribution of the axial parenchyma cells among other xylem elements is important from the taxonomical point of view, while also giving additional information about the function of living cells in wood. Axial parenchyma cells rarely occur in gymnosperms, whereas in angiosperms they are more frequent and can form various patterns, e.g., in *Alnus glutinosa* or *Crataegus* sp., where parenchyma cells are sparsely localized, while in *Ficus* sp., they are

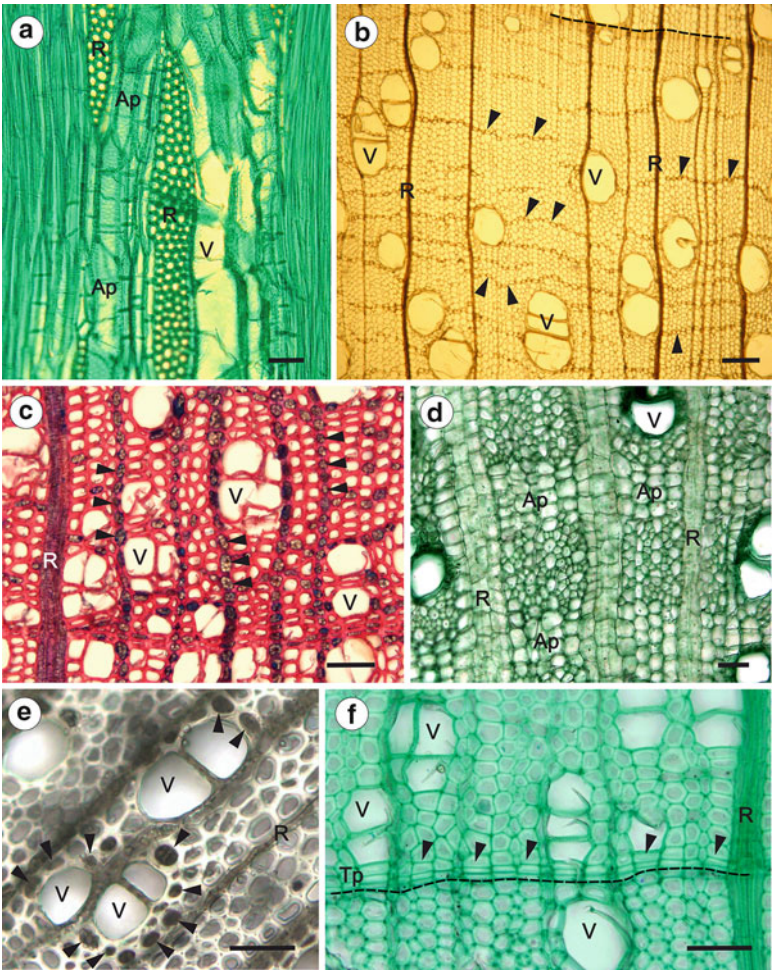

Fig. 4.3 The axial system of xylem parenchyma in angiosperms. (**a**) Strands of axial terminal parenchyma cells are shown in the tangential section of the secondary xylem of *Ulmus* sp. The cells have tapered ends and maintain the shape of fusiform cambial initials, from which they derived. (**b**–**f**) Different types of apotracheal (**b**–**d**, **f**) and paratracheal (**e**) axial parenchymas are presented in transverse sections of *Juglans* sp. (**b**), *Cornus* sp. (**c**), *Ficus* sp. (**d**), *Acer* sp. (**e**), and *Betula* sp. (**f**) wood. Axial parenchyma cells are marked by *arrowheads* on microphotographs (**b**), (**c**), (**e**), and (**f**). (**b**–**d**) In the apotracheal type, narrow (**b**, **c**) or wide (**d**) strands of axial parenchyma cells are distributed perpendicular (**b**, **d**) or parallel (**c**) to the xylem rays. (**e**) In the paratracheal type, axial parenchyma cells are closely associated with vessels. (**f**) Terminal parenchyma, the characteristic type of apotracheal parenchyma, is formed at the end of growing season and it is localized alongside the border of annual ring. *Ap* axial parenchyma, *R* xylem ray, *Tp* terminal parenchyma, *V* vessel; *dashed line* shows the border of annual rings. Microphotographs were taken in white transmitted light, scale bars: 50 μm (**a**, **b** courtesy of Dr. E. Myśkow)

abundant and form wide bands of living cells in the xylem region (Fig. 4.3; Metcalfe and Chalk 1983; Carquist 1988; IAWA 1989). Two major types of axial parenchyma—apotracheal and paratracheal—can be distinguished based on the type of cell distribution in regard to vessels (Fig. 4.3). In paratracheal parenchyma, the most common in angiosperms, the living cells surround tracheary elements and form an incomplete or complete sheath around the vessels. In the case of apotracheal types, the cells of axial parenchyma are distributed without relation to the vessels, being usually sparsely localized in the xylem region. When apotracheal parenchyma cells are more frequent, they can aggregate, forming smaller groups or bands among the other xylem elements. Moreover, axial parenchyma cells can be closely associated with growth rings, forming narrow bands at the beginning (initial parenchyma) and at the end (terminal parenchyma) of the growing season (Fig. 4.3). The latter type is clearly more frequent in woody plants (Braun and Wolkinger 1970; Metcalfe and Chalk 1983; Carquist 1988; IAWA 1989).

4.2.3 Xylem Parenchyma Cells: Classification Based on Cell Function

The cells of both axial and radial xylem parenchyma play different functions in sapwood. They can form storage tissue, where accumulation and remobilization of the storage substances take place, or they can serve as specialized cells which are in contact with conducting elements and are involved in exchange at the symplasm/apoplasm interface (Czaninski 1977; Barnett 2006).

The terminology of the latter, specialized xylem parenchyma cells, is diverse. Generally, these cells are described as "accessory tissue" that is formed by parenchymatous cells closely associated through numerous pits with the tracheids or vessels. Accessory tissue includes the contact cells of xylem rays and paratracheal parenchyma cells abutting the tracheids in gymnosperms and vessels in angiosperms (Braun 1984). According to another, more narrow classification, "contact cells" are defined as specialized groups of living axial and radial xylem parenchyma cells that are not only contiguous with tracheary conducting elements but are particularly involved in solute exchange between the symplasm and apoplasm in wood (Gregory 1978). In angiosperms, a narrow term of "vessel-associated cells," which refers to the xylem parenchyma cells facing the vessels and physiologically linked with them, was proposed by Czaninski (1977). Alternatively, it has been suggested to maintain the term "contact cells" as a broader and more accurate definition, true for all seed plants (Gregory 1978). In recent literature both expressions are used frequently and alternatively, because the biological functions of contact cells in seed plants and vessel-associated cells in angiosperms are similar. In this chapter the term "contact cells" will be used and refer to all seed plants.

4.3 Cellular Organization of Xylem Parenchyma Cells

4.3.1 Metabolic Activity and Vitality

Xylem parenchyma is composed of long-living cells which are metabolically active during the whole year (Fig. 4.4). Judging by cell respiration, the metabolic rate is estimated to be slightly higher in xylem parenchyma cells than in storage tissues,

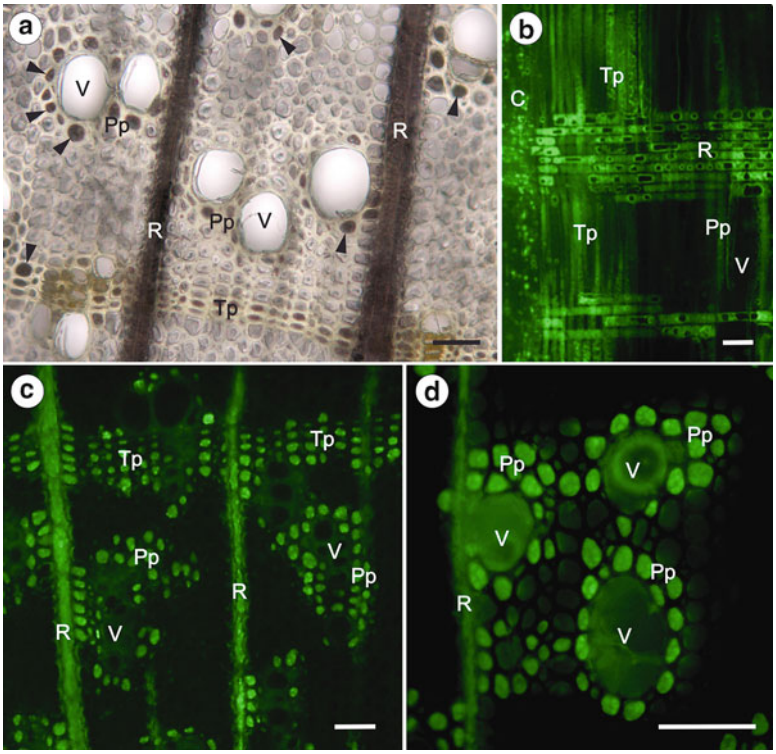

Fig. 4.4 The metabolic activity and symplasmic coupling of xylem parenchyma cells in the sapwood of *Acer pseudoplatanus*. (**a**) The metabolic activity of xylem parenchyma cells was confirmed by staining with thiazolyl blue. The dark color of formazan, the product of mitochondrial dehydrogenases activity in thiazolyl blue reaction (marked with *arrowheads*), is visible in the xylem rays and in the cytoplasm of paratracheal and terminal axial parenchyma cells. (**b**–**d**) Symplasmic connectivity between living xylem parenchyma cells visualized by carboxyfluorescein (CF). After loading of carboxyfluorescein diacetate solution into the maple branches through the vascular system and its conversion to fluorescent tracer—CF in the living cells—the dye was transported symplasmically within the spatial system of living cells in the secondary xylem. The strong fluorescent signal of CF in the cytoplasm of xylem rays and paratracheal and terminal axial parenchyma makes visible the routes of symplasmic transport in sapwood. *C* cambium, *Pp* paratracheal parenchyma, *R* xylem ray, *Tp* terminal parenchyma, *V* vessel; *arrowheads* show metabolically active axial parenchyma cells. Microphotographs were taken in white transmitted light (**a**) and in blue (475 nm) excitation light (**b**–**d**) in the epi-fluorescent microscope, scale bars: 50 μm

but far below the meristematic cells. The respiration activity of xylem parenchyma is low and varies between tree species; however, it does not decline with cell age (Spicer and Holbrook 2007).

The vitality of parenchyma cells subsequently lowers with the increase in distance from the cambial region and can be exemplified by decreasing starch content and the number of organelles in the ray cells towards the center of the trunk (Höll 1975; Song et al. 2011), although cell vitality is not a simple function of the distance from the cambial region. Among the population of living xylem parenchyma cells, some of them can die earlier, while others later. This discrepancy can be induced by the process of early cell death that occurs in sapwood when cells have contact pits with short-living conducting elements of the xylem, e.g., ray tracheids in *Pinus* and *Abies* (Nakaba et al. 2006, 2008) or vessels in *Populus* (Nakaba et al. 2012a). The occurrence of early cell death is species dependent. No evidence of natural early cell death, either in ray or in axial parenchyma, was found in the sapwood of *Acer rubrum*, *Fraxinus americana*, or *Quercus rubra*, where all analyzed parenchyma cells were alive (Spicer and Holbrook 2007). Nevertheless, during the process of heartwood formation, all xylem parenchyma cells ultimately die (see Sect. 4.4.3.4). The timing of cell death during heartwood formation varies between different types of xylem parenchyma. It begins earlier in axial parenchyma, compared to the rays, and depends on vicinity to the pith region as was shown in branches of *Robinia pseudoacacia* (Nakaba et al. 2012b).

4.3.2 Arrangement of the Cytoskeleton Network

Long-living xylem parenchyma cells exhibit a characteristic organization of the cytoskeleton. Microtubules and microfilaments are bundled and show a generally net axial orientation (Chaffey and Barlow 2001, 2002). They spread from one end of the cell to the other and are parallel to the long axis of prosenchymatous xylem parenchyma cells. Thus, according to cell shape and in respect to the main axis of the stem, cytoskeleton components are arranged radially in ray cells and vertically in axial parenchyma, although a meshwork arrangement of microfilaments or helical arrays of microtubules can also be found (Chaffey et al. 1997, 1999, 2000; Chaffey and Barlow 2001, 2002). The mostly axial distribution of the cytoskeleton is disturbed in the pit periphery, where concentric rings of microfilaments and microtubules are present (Chaffey and Barlow 2002; see Sect. 4.3.4). The directional orientation of microtubules and microfilaments may facilitate intracellular transport and assist in the intercellular movement. Additionally, the cytoskeleton network in radial and axial xylem parenchyma cells forms a continuity with similar cytoskeleton elements of cambial and phloem regions, creating a three-dimensional system supporting symplasmic transport in plants (Chaffey and Barlow 2001, 2002).

4.3.3 Characteristics of the Cell Wall

Xylem parenchyma cells are usually surrounded by a thick primary cell wall and a thick, frequently multilayered, secondary cell wall. The latter is generally rich in lignins and pectins (Chafe and Chauret 1974). The lignification process starts earlier in the contact cells than in the isolation cells, with the exception of pit membranes, which are composed of a primary cell wall and are clearly unlignified for a very long time (Chafe and Chauret 1974; Chafe 1974; Murakami et al. 1999; Nakaba et al. 2012a). Formation of the secondary cell wall and deposition of its component, xylan, start much later in ray cells than in fibers and vessels (Kim et al. 2012). Additionally, in some conifers, considerably delayed lignification or the lack of a secondary cell wall is characteristic of ray parenchyma cells that are not in direct contact with ray tracheids. These ray parenchyma cells can maintain the primary nature of the cell wall for more than 30 years, as in *Pinus densiflora*, while in *Pinus rigida* a secondary cell wall does not form in these cells at all (Nakaba et al. 2008).

Parenchyma cells neighboring to the tracheary elements have an additional layer of a loose-textured, usually unlignified material that is generally called the "protective layer" (Chafe 1974; Mueller and Beckman 1984; Wiśniewski et al. 1987; Barnett et al. 1993). The protective layer extends between plasmalemma and the entire inner surface of the secondary cell wall of contact cells. It is usually thicker around the pits (Chafe 1974; van Bel and van der Schoot 1988; Barnett et al. 1993). The real role of the protective layer is still a matter of debate. This layer was believed to protect living paratracheal cells against high osmotic pressure and the oscillations present in the conducting elements of the xylem (van Bel and van der Schoot 1988). However, it is not clear why some cells, e.g., ray cells, without this layer can survive in contact with dead xylem elements (Chafe 1974; Chafe and Chauret 1974) or how a layer formed mainly by pectins and cellulose can withstand hydrostatic fluctuations in conducting tracheary elements (Barnett et al. 1993). Nor is the protective layer necessary for formation of tyloses—outgrowths of parenchyma cells into tracheary elements—as it can occur in non-tylose-forming species and be absent in tylose-forming ones (Chafe and Chauret 1974; Barnett et al. 1993). Presently it is suggested that the protective layer is not involved in plant defense but rather in intercellular exchange at the symplasm/apoplasm interface (Barnett et al. 1993). Due to the low permeability of secondary cell walls, demonstrated in experiments with lanthanum (Wiśniewski et al. 1987), the protective layer can increase continuity between the protoplast and the lumen of tracheary elements (Barnett et al. 1993). Its presence increases the surface of the symplasm/apoplasm interface, which is not limited to the pit regions exclusively but expands to the entire plasmalemma surface of the contact cells (Barnett et al. 1993). Thus, the protective layer considerably enhances the efficiency of intercellular exchange in woody plants.

4.3.4 Pitting and Plasmodesmata Distribution

The striking differences in the permeability of pit membranes compared to secondary cell walls suggest that the longitudinal and lateral exchange of solutes in the xylem region occurs via the pit system (Wiśniewski et al. 1987). Pits between xylem parenchyma cells are usually simple, while half-bordered at contact with tracheary elements (Yang 1978). The type of pit—simple or bordered—strongly depends on the thickness of xylem parenchyma walls (Yang 1978; Carquist 2007). Simple pits are localized in relatively thin walls. As the thickness of the secondary cell wall increases, selective pressure rises to form bordered pits. Thus, bordered pits among living parenchyma cells, especially in ray cells, are quite common (Carquist 2007).

The distribution and density of the pits provide important information about the possible direction and intensity of solute flow between the cells. Pits are usually unevenly distributed, e.g., in tangential walls which are more densely pitted compared to radial walls in procumbent ray cells, while in square and upright ray cells, the pits are more or less equally distributed through all cell walls (Carquist 2007). The function of contact cells in the transfer between symplasm and apoplasm is underscored by the presence of extremely large pits between tracheary elements and contact cells (Fig. 4.2; Sauter et al. 1973; Sauter and Kloth 1986; Murakami et al. 1999).

Pits occurring between living parenchyma cells have a characteristic ultrastructure, with concentric rings of both microtubules and microfilaments, as can be seen by the immunolocalization of myosin, tubulin, and actin around a pit area (Chaffey and Barlow 2002). During pit formation, microfilaments probably prevent the deposition of the secondary cell wall at the pit membrane by exclusion of microtubules from the pit periphery (Chaffey and Barlow 2002). Thus, the pit membranes of xylem parenchyma cells are thin and possess the primary structure of a wall, with numerous simple or branched plasmodesmata (Yang 1978; Sauter and Kloth 1986; Wiśniewski et al. 1987; Chaffey and Barlow 2001).

Besides living cells, plasmodesmata are also frequently present in the pseudo-tori, which are thickenings of the primary cell wall located eccentrically in the developing pit membranes of fibers, fiber tracheids, and vessels of some angiosperm families such as Rosaceae and Ericaceae (Barnett 1982, 1987a, b; Lachaud and Maurousset 1996; Rabaey et al. 2006, 2008). Plasmodesmata in pseudo-tori are densely packed, branched, and secondary in origin (Barnett 1982, 1987a, b; Rabaey et al. 2006; Jansen et al. 2007). Just before accomplishing cell differentiation and protoplast autolysis, a secondary cap-like layer is deposited on the primary thickenings of the pseudo-tori (Barnett 1987a; Lachaud and Maurousset 1996; Jansen et al. 2007; Rabaey et al. 2006, 2008). This additional layer can seal intercellular connections, but plasmodesmata can sometimes protrude through its outer part (Lachaud and Maurousset 1996; Rabaey et al. 2008). It is unknown whether or not the plasmodesmata in pseudo-tori are functional. The irregular and amorphous shape of pseudo-tori, as well as an eccentric position that does not match the pit aperture, suggests that pseudo-tori cannot properly seal the pits and thus they are not involved in pit

aspiration (Rabaey et al. 2006, 2008). Therefore, it is possible that the pseudo-tori are formed to maintain intercellular communication between various xylem elements (Barnett 1982, 1987b; Lachaud and Maurousset 1996; see Sect. 4.4.3.5).

4.4 Functions of Xylem Parenchyma Cells in the Secondary Xylem

The long-living xylem parenchyma, which is composed of both axial and radial systems, forms a unique, functional continuum for efficient intercellular communication (Fig. 4.5). Thus, the xylem parenchyma is a vital component of the secondary

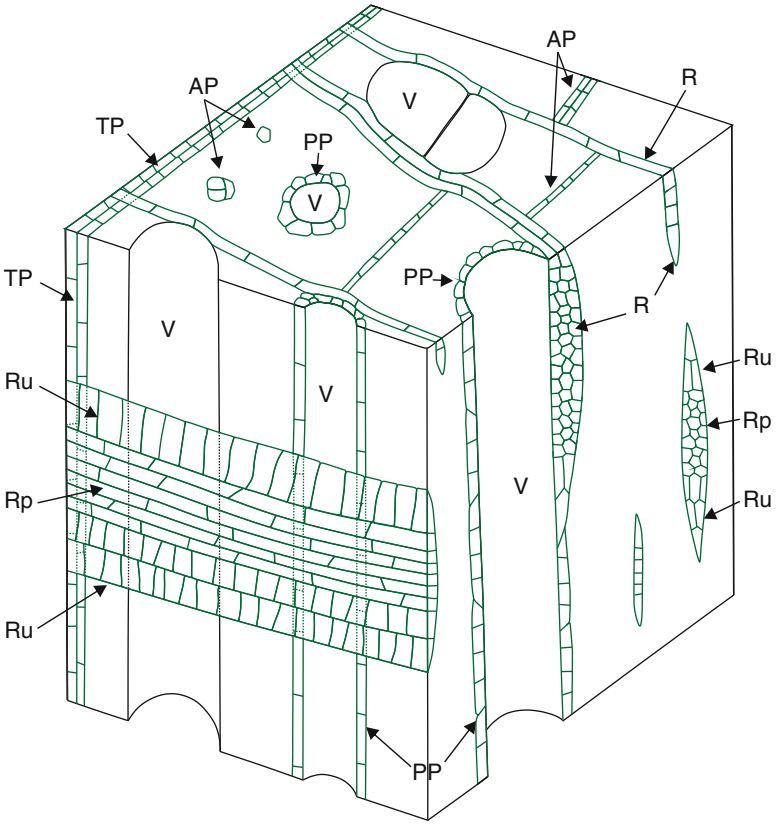

Fig. 4.5 Schematic diagram of living parenchyma cells in the secondary xylem of angiosperms. Xylem rays and different types of axial apotracheal and paratracheal parenchymas form the spatial system of interconnected living cells in sapwood. To simplify the diagram, only vessels are indicated, although other types of nonliving elements (tracheids, fibers, fiber tracheids) are also present in the secondary xylem. *Ap* apotracheal parenchyma, *Pp* paratracheal parenchyma, *R* xylem ray, *Rp* procumbent xylem ray, *Ru* upright xylem ray, *Tp* terminal parenchyma, *V* vessel

xylem. The whole spatial network of interconnected and integrated cells is an example of a three-dimensional symplasmic field where free symplasmic short- and long-distance transport is continued (Figs. 4.4 and 4.5). Apart from the main function of storing and distributing reserve substances, living xylem parenchyma cells are involved in exchange at the symplasm/apoplasm interface, embolism repairs, defense mechanisms against vascular pathogens, and vascular structure (Chowdhury 1953; Sauter 1982; Benhamou 1995; Hilaire et al. 2001; Améglio et al. 2004; Salleo et al. 2004). They also play an important role in the biomechanics of living deciduous trees and increase radial strength and the transverse elastic anisotropy of trunks (Burgert et al. 1999, 2001; Burgert and Eckstein 2001).

Wood without living cells cannot exist. Even in a species devoid of rays, at least in juvenile wood, living cells of axial parenchyma and nucleated fibers are present (Barghoorn 1941b; Lev-Yadun and Aloni 1991; Carquist 2009, 2010). Over the course of evolution, the volume of living parenchyma cells in the secondary xylem and their specialization have increased. Initially, the diffusive parenchyma cells supported the conductive system by maintaining the appropriate water column in vessels. Then, the tendency to group living axial elements around the vessels, together with the intensive secondary thickening of trunks, forced the integration of axial parenchyma cells with developing rays. At the end, due to the widening of the rays and the predominance of procumbent cells, the rays became a route for photoassimilate transport across the stem (Carquist 2009).

4.4.1 Storage Parenchyma

Storage parenchyma is specialized for seasonal accumulation and mobilization of organic and inorganic materials. Besides starch and lipids (the most frequent storage materials in plants) parenchyma cells can store nitrogen compounds in the form of protein bodies as well as crystals and silica bodies (Sinnott 1918; Ziegler 1964; Höll 1975; Carquist 1988; Harms and Sauter 1992a). Starch grains are accumulated mostly in the apotracheal parenchyma and in isolation xylem ray cells (Gregory 1978), as well as in the living fibers of the outermost ring (Yamada et al. 2011). The amount of accumulated starch grains varies among different types of xylem parenchyma cells and depends on the distance from the cambium, where the largest quantity of starch is deposited in the current xylem year (Nakaba et al. 2012a). Parenchyma cells have also a protein-storing function. In the small vacuoles of temperate-zone trees, polypeptides of 32–36 kDa are accumulated and form specific protein bodies. They appear near the cambium, mainly in the rays and in axial paratracheal parenchyma (Harms and Sauter 1992a, b; Hao and Wu 1993).

The quantity of accumulated storage compounds in xylem parenchyma changes seasonally. It corresponds to plant activity and to alternating periods of nutrient abundance and deficiency. Reserves are usually accumulated at the end of a growing season and then utilized in spring time, when photoassimilates are necessary but are not yet produced (Harms and Sauter 1992a; Sauter and van Cleve 1994; Sauter 2000).

For example, the starch content in poplar and willow trees is lowest after budbreak and leaf expansion. Then, the starch amount starts increasing and reaches maximum value in autumn, when the leaves are shed. After that, starch content gradually decreases (Essiamah and Eschrich 1985; Sauter et al. 1996). The lowered level of starch in winter correlates with increased amounts of sugars (Sauter and van Cleve 1994) which are involved in the adaptation of the cells to frost and in the refilling of embolized tracheary elements (Essiamah and Eschrich 1985; Sauter et al. 1996). Starch is resynthesized again from available sugars before spring. Its content rises temporarily, until the budbreak period, when reserves are utilized during plant growth (Sauter 1972; Essiamah and Eschrich 1985; Harms and Sauter 1992b; Sauter and van Cleve 1994; Sauter et al. 1996; Sauter 2000). Similarly, the amounts of other stored compounds can change seasonally. Lipids are intensively accumulated in summer and remain at a prominent level during the entire winter until spring remobilization (Sauter and van Cleve 1994). However, protein bodies are formed mostly in fall, in the time of leaf yellowing, and are almost completely degraded during budbreak (Sauter and van Cleve 1990; Harms and Sauter 1992a). Nitrogen released from protein bodies is used during the onset of new shoot growth (Langheinrich and Tischner 1991).

Due to the seasonally regulated quantity of storage compounds, the ultrastructure of storage parenchyma cells changes dramatically during the year. Before budbreak the protoplasts of storage parenchyma contain numerous amyloplasts, oleosomes, and protein bodies (Harms and Sauter 1992a, b; Sauter and van Cleve 1994; Sauter et al. 1996). After the onset of new growth in spring, the number of starch grains is significantly reduced, oleosomes almost disappear, while protein bodies can no longer be detected (Harms and Sauter 1992a).

4.4.2 Long-Distance Transport Routes in Woody Stems

The seasonally alternating accumulation and mobilization of storage compounds in xylem and phloem parenchyma cells requires precisely controlled translocation of reserves to the sink regions. This has been clearly demonstrated in experiments with dormant cambium, in which local heating of the stem induced division activity in the cambial cells and starch remobilization from the phloem towards the cambium (Oribe et al. 2001, 2003; Begum et al. 2007). In sapwood, the manner of photoassimilate and other solute flow in the living xylem cells is still a matter of debate (van Bel 1990; Sauter 2000). Although the long-distance transport of water and solute minerals in the apoplasmic system via dead xylem elements like tracheids and vessels is unquestionable (Holbrook and Zwieniecki 2005), the structure of long-living axial and radial parenchyma cells (Fig. 4.5) suggests that communication and trafficking via the symplasmic route is likewise important.

The shape of the xylem parenchyma cells and pit distribution localized in their cell walls (see Sect. 4.3.4) reflect the major directions of solute flow. Axial parenchyma cells are involved in vertical transport, procumbent ray cells in radial

spreading, while square to upright ray cells represent an intermediate step (Carquist 2007). Among the specific ray cells abutting the vessels, isolation cells are involved in radial transport and function also as a storage region (Sinnott 1918; Sauter 1972; Sauter and Kloth 1986; Murakami et al. 1999). Due to larger pit-field areas and a higher frequency of plasmodesmata at the tangential walls, isolation cells are better specialized for radial symplasmic transport than contact cells (Sauter and Kloth 1986; Sauter 2000). However, intercellular spaces between isolation cells (Murakami et al. 1999) may also suggest the involvement of the apoplasmic transport route. Delayed differentiation of the secondary cell wall in isolation cells, as compared to contact cells, suggests that the unlignified tangential walls can temporarily assure apoplasmic continuity between phloem and xylem (Murakami et al. 1999). However, radial trafficking in apoplasm, as demonstrated using specific tracers like acid fuchsin and safranin, seems to be limited. These apoplasmic dyes loaded to poplar plants through the vascular system were only visible in the close vicinity of vessel elements and did not move further in the cell wall system of ray parenchyma cells (Sano et al. 2005). Additionally, the simultaneous application of apoplasmic (sulforhodamine B, SRB) and symplasmic (carboxyfluorescein, CF) tracers to maple tree branches revealed a difference in efficiency between both routes of transport. Radial apoplasmic transfer of SRB was restricted to the axial xylem elements, especially vessels, while at the same time CF was visible in the symplasm of xylem rays and in the cambial region (Sokołowska and Zagórska-Marek 2012), suggesting a prevailing role of symplasmic route in radial transfer in wood.

4.4.2.1 Radial Symplasmic Movement

Xylem and phloem rays function as living bridges that connect the cambial region and living elements of secondary xylem and phloem. Therefore, they are involved in centripetal and centrifugal radial symplasmic transport across the stem (Langenfeld-Heyser 1987; van Bel 1990; Sokołowska and Zagórska-Marek 2007, 2012). This route of transport is facilitated by abundant plasmodesmata present in the tangential walls of the rays (Barnett 1982; Sauter and Kloth 1986) and was demonstrated in experiments with tracer loading (Fig. 4.4) and membrane potential measurement (van der Schoot and van Bel 1990; Sokołowska and Zagórska-Marek 2007, 2012; Fuchs et al. 2010). Moreover, radial translocation of ^{14}C-labeled assimilates (Langenfeld-Heyser 1987; Korolev et al. 2000b) and high translocation rates of sugars through the tangential area of ray cells (Sauter and Kloth 1986; Sauter 2000) strongly support the relevance of the radial symplasmic pathway across the stem and towards the cambium. Furthermore, localization of storage proteins remobilized during the first divisions of cambial cells in *Populus* directly confirmed the significance of the radial symplasmic route. Reserves can indeed be transported symplasmically between phloem parenchyma cells, ray cells, and fusiform cambial cells, because proteinaceous particles were clearly localized in the cytoplasmic sleeve of plasmodesmata (Fuchs et al. 2010).

4.4.2.2 Intercellular Spaces: Apoplasmic Route for Gas Exchange

A complex system of intercellular spaces spans along the axial and radial groups of parenchyma cells, from cambium towards the mature xylem, and serves as an additional apoplasmic route of transport in secondary xylem (van der Schoot and van Bel 1989; Sun et al. 2004; Nagai and Utsumi 2012). This system is comprised of short vertical and prominent radial canacules that probably originate from fusion of schizogenous spaces (van der Schoot and van Bel 1989).

Intercellular spaces are separated from living ray cells by blind pits and seem to be filled with water (van der Schoot and van Bel 1989) or with gases (Nagai and Utsumi 2012). Thus, they are considered to be involved in water (van der Schoot and van Bel 1989; van Bel 1990; Sun et al. 2004; Kitin et al. 2009) or gas (Hook and Brown 1972; Spicer and Holbrook 2005; Nagai and Utsumi 2012) trafficking, presumably in a radial direction. However, the transfer of water and solutes via intercellular spaces has recently been questioned (Nagai and Utsumi 2012). Firstly, intercellular spaces are prominent in tree species living in wet habitats and having a higher tolerance to flooding, e.g., in *Nyssa aquatica* and *Fraxinus pennsylvanica*, where their presence probably increases plant aeration (Hook and Brown 1972; Spicer and Holbrook 2005). Secondly, in conifers, intercellular spaces stay empty even if they surround living rays and tracheids filled with cytoplasm or water (Nagai and Utsumi 2012). Thus, it seems plausible that intercellular spaces function rather as a gas exchange system in apoplasm (Nagai and Utsumi 2012).

4.4.2.3 The Role of Cambial Cells in Radial Xylem–Phloem Exchange

Cambium serves as a strong sink that draws nutrients due to a high level of division activity and production of derivatives (Krabel 2000; Korolev et al. 2000b). The radial transport of ^{14}C-assimilates (Langenfeld-Heyser 1987; Tomos et al. 2000; Korolev et al. 2000a, b) and fluorescent tracers (van der Schoot and van Bel 1990; Sokołowska and Zagórska-Marek 2007) from the phloem and xylem regions to the cambium occurs symplasmically and seems to be partially ceased at the cambial zone. The symplasmic route of transport towards the cambium corresponds well with the general principle that unloading to the meristematic tissues or growing organs is symplasmic (Patrick and Offler 1996; Tarpley and Vietor 2007). Moreover, it seems that cambial meristem collects assimilates and metabolizes them quickly to obtain energy for functioning and division activity (Krabel 2000; Korolev et al. 2000a), because cambial cells reveal very low concentrations of K^+ and sugars (sucrose, glucose, fructose) compared with neighboring cells of the phloem and xylem regions (Korolev et al. 2000a; Tomos et al. 2000). Accordingly, it becomes obvious why dormant cambial cells, experimentally activated by local heating, cannot divide further in the event of deficiency of reserve materials (Oribe et al. 2001, 2003).

4.4.2.4 Symplasmic Transport: Preferential Route of Transport via Parenchyma Cells in Sapwood

To summarize, the anatomical and physiological features of living xylem parenchyma cells, such as plasmodesmata distribution and frequency (Sauter and Kloth 1986; Barnett 2006), cytoskeleton organization (Chaffey and Barlow 2001, 2002), storage substances mobilization (Fuchs et al. 2010), and movement of fluorescent tracers (Fig. 4.4; Sokołowska and Zagórska-Marek 2012), evidence the role of symplasmic trafficking via parenchyma cells in xylem and towards the cambium. Although, the final choice of the route in xylem parenchyma—symplasmic or apoplasmic—depends mostly on the type of substance transported (Sharkey and Pate 1975; Vogelmann et al. 1985). However, bearing in mind the restricted radial trafficking of apoplasmic tracers and the involvement of intercellular spaces in gas exchange, it can be considered that intercellular communication via living xylem cells in sapwood occurs prominently via the symplasmic route (Fig. 4.5).

4.4.3 Contact Cells: Specialized Types of Axial and Radial Parenchyma Cells

Contact cells, described in angiosperms as vessel-associated cells, are usually small, living parenchyma cells abutting tracheary elements. They have a dense cytoplasm with a voluminous nucleus, small vacuoles, and numerous ribosomes and mitochondria (Czaninski 1977; Alves et al. 2001, 2007). Starch is usually absent, even during the winter season (Sauter et al. 1973; Czaninski 1977; Braun 1984). In trees of temperate zones, starch appears in contact cells only temporarily—in early spring—and is quickly remobilized just before and during bud swelling, considerably earlier than in the neighboring cells of typical storage parenchyma (Sauter 1972; Sauter et al. 1973; Braun 1984). Contact cells are involved in varied physiological processes due to their bidirectional transmembrane transport ability at the symplasm/apoplasm interface. Sugar influx and efflux (Sauter 1982), exudation of occluding and toxic substances during vascular pathogen infection (Shi et al. 1992; Benhamou 1995; Cooper et al. 1996) and of phenolic compounds during heartwood formation (Magel et al. 1991; Spicer 2005), require a controlled exchange of substances between contact cells and tracheary elements, via active and passive routes.

Despite the contact cells being directly connected with tracheary elements by large and densely packed contact pits (Fig. 4.2; Sauter 1972; Sauter et al. 1973; Murakami et al. 1999), they are devoid of plasmodesmata in contact pit membranes (Czaninski 1977; Decourteix et al. 2006), and thus they are symplasmically isolated from vessels and tracheids. Therefore, communication and trafficking between the apoplasm, especially the xylem sap of tracheary elements and contact cells as a part of three-dimensional continuum of living xylem parenchyma cells, have to proceed through the plasma membrane. Probably, the interface of symplasm and apoplasm

is enhanced by a protective layer in contact cells (see Sect. 4.3.3), which maintains apoplasmic continuity with vessels.

The intensive transmembrane transport at the symplasm/apoplasm interface requires the increased metabolic activity of contact cells (Fig. 4.4). And indeed, contact cells exhibit enhanced levels of acid phosphatases, peroxidase, and dehydrogenases, in comparison to storage parenchyma cells (Sauter 1972; Sauter et al. 1973; Braun 1984; Alves et al. 2001). The enzymatic activities of contact cells change seasonally in trees of the temperate zones. They increase in late fall and winter to maximum in spring time, before and during the budbreak period, then cease rapidly in late spring when leaves are flushing, to finally become undetectable throughout the entire summer (Sauter 1972; Sauter et al. 1973). The high metabolic activity of contact cells is closely related to the low level of starch in these cells, and starch resynthesis coincides with a rapid drop of acid phosphatase activity (Sauter 1972; Sauter et al. 1973). Thus, it seems reasonable that in continuously growing tropical trees, contact cells are free of starch and show permanent acid phosphatase activity (Braun 1984). Regarding the high metabolic activity of contact cells with simultaneous lack of starch, it can be suggested that these cells are an important site of respiratory activity and are involved in intensive transport between apoplasm and symplasm (Sauter 1972; Gregory 1978; Murakami et al. 1999; Alves et al. 2001).

4.4.3.1 Sugar Influx/Efflux Interactions at the Symplasm/Apoplasm Interface

Sucrose content in the tracheary sap of temperate zone trees changes throughout the year, even in winter time when the growth activity of plants in restricted (Sauter 1980, 1982, 1983, 1988; Sauter et al. 1996; Améglio et al. 2001, 2004). Sucrose can be released from starch; thus, the amount of sucrose is inverse to starch content and changes seasonally. Accumulation of sucrose in tracheal sap usually starts at the beginning of winter, reaches its maximum in midwinter, keeps high values before and during budbreak period, and drops sharply when leaves are flushing (Sauter 1982, 1983, 1988; Améglio et al. 2002). The high osmolarity of tracheal sap coincides with drop of starch concentration in storage parenchyma and the high metabolic activity of contact cells, indicating the involvement of the contact cells in sucrose efflux into tracheary elements (Sauter 1972, 1980, 1981, 1982, 1983, 1988; Sauter et al. 1973; Améglio et al. 2004). In winter, sugar efflux is a slow continuous and selective process that depends on many factors such as temperature, respiration, sucrose concentration in xylem parenchyma cells, the intensity of starch hydrolysis and resynthesis, as well as the rate of sugar absorption into the symplasm (Sauter et al. 1973; Sauter 1981, 1982, 1988; Améglio et al. 2001, 2004). Efflux cannot operate as a simple leaking into the apoplasm, but probably occurs by facilitated diffusion mediated by a yet unknown protein, and regulated by metabolic inhibitors (Sauter 1980, 1982, 1988; Améglio et al. 2004). Alternatively, sugar efflux has recently been proposed as an energy-consuming process that is mediated by proton co-transporters (Salleo et al. 2004; Secchi and Zwieniecki 2012). Apart from sugar

efflux, contact cells are also involved in an opposite process of active sugar influx from tracheary sap to symplasm (Sauter 1983; Decourteix et al. 2006, 2008; Alves et al. 2007; Bonhomme et al. 2009). Influx is carried out by putative xylem sucrose and hexose transporters (Decourteix et al. 2006, 2008) which depend on the driving force generated by H^+-ATPase localized in the plasma membrane of contact cells (Fromard et al. 1995; Arend et al. 2002, 2004; Decourteix et al. 2006; Alves et al. 2007). Which of these two competing biochemical processes dominates, sugar efflux or influx, depends mostly on temperature, and thus it is periodically regulated (Sauter 1982, 1983; Améglio et al. 2004; Decourteix et al. 2006; Alves et al. 2007; Bonhomme et al. 2009). Efflux dominates in midwinter, when temperatures are low (below 5 °C). In that period sugars are converted from starch in the xylem parenchyma cells and then accumulated in tracheary elements (Sauter 1980, 1982, 1983; Sauter et al. 1996; Alves et al. 2007; Salleo et al. 2009). The activity of H^+-ATPase is highly reduced at low temperatures (Alves et al. 2001, 2007; Améglio et al. 2004), resulting in the cessation of sugar influx and, in turn, in unlimited sugar efflux to apoplasm. When temperatures are higher (over 15 °C), starch begins to resynthesize. The sucrose concentration gradient lowers at the symplasm/apoplasm interface and decreases the efflux rate (Sauter 1983, 1988; Sauter et al. 1996). Additionally, H^+-ATPase is highly active and produces the proton motive force used by transporters for active sugar uptake. Thus, at higher temperatures in winter, sugar influx dominates (Sauter 1982, 1983, 1988; Alves et al. 2001, 2007; Améglio et al. 2004; Bonhomme et al. 2009).

4.4.3.2 The Biological Role of Active Substance Secretion and Uptake by Contact Cells

Contact cells are involved in specialized translocation of water and other materials between symplasm and apoplasm (Gregory 1978; Murakami et al. 1999; Salleo et al. 2004; Decourteix et al. 2006; Secchi and Zwieniecki 2011), although the detailed mechanism of sugar exchange between contact cells and tracheary elements seems to be more complex (Alves et al. 2007; Nardini et al. 2011; Secchi and Zwieniecki 2011, 2012). Sucrose accumulation in the tracheary sap of *Acer saccharum* (sugar maple) from November to April, which coincides with the high metabolic activity of contact cells in the vicinity of functional, water-conducting elements and a low level of starch content, together suggest that contact cells are able to release sugars and generate high osmotic pressure in vessels (Sauter 1972; Sauter et al. 1973; Braun 1984). Thus, they can facilitate water transport in xylem elements and ascend the sap before leaf flushing in early spring (Braun 1984). This can be particularly important in tropical trees during conditions of lowered transpiration due to high humidity during overcast days (Braun 1984). A dramatic increase in water uptake in early spring was recorded in some trees like *Acer pseudoplatanus*, *Betula pendula*, *Alnus glutinosa,* or *Fagus sylvatica*, although the origin of the spring sap was not determined (Essiamah and Eschrich 1986). In at least a few tree

species, e.g., *Juglans regia* (walnut), the increased positive xylem pressure in spring and autumn is mainly generated by root pressure (Ewers et al. 2001). Thus, the mechanism responsible for the rising of sap in spring time varies between species and requires further investigation.

Xylem elements are an efficient translocation route for organic compounds to the meristematic tissues in species which flower before leaf appearance, in late winter or early spring, when the phloem strands are still nonfunctional (Sauter 1980, 1983). For instance, sugar influx rate and the activity of H^+-ATPase in spring time are considerably higher in apical parts of 1-year-old branches, bearing bursting buds in walnut, compared to basal stem regions with non-bursting buds (Alves et al. 2007). Over 90 % of the total sucrose pool is provided for the opening of apical buds by xylem elements. This means that the xylem system is practically the only source of sucrose in the apical parts of walnut during budbreak (Bonhomme et al. 2009). This emphasizes the importance of contact cells in sugar uptake from apoplasm during growth resumption (Alves et al. 2007). Further translocation of sugars away from contact cells probably occurs via plasmodesmata, due to symplasmic connectivity between contact cells and the neighboring storage parenchyma (see Sect. 4.4.2). Interestingly, meristematic regions and vascular strands are symplasmically coupled (Duckett et al. 1994; Gisel et al. 1999; Banasiak 2011). Therefore, continued transport via living cells and the elements of the primary vascular system towards the apices serve as an interesting hypothesis that requires further examination. The most surprising fact related to sugar influx in walnut stems is the significantly low active uptake, reaching only 50 % of global sugar uptake in the basal parts of branches, where most of buds do not break (Bonhomme et al. 2009). Thus, different pathways are necessary in basal stem regions for sugar uptake from apoplasm. Although facilitated diffusion is possible (Bonhomme et al. 2009), an alternative route via endocytosis seems to be more convincing. It has previously been suggested that uptake of membrane-impermeant fluorescent tracers—Lucifer Yellow and Texas Red-labeled dextran (10 kDa)—by xylem parenchyma cells abutting the vessel in rice leaves occurs via endocytosis (Botha et al. 2008). Thus, contact cells are crucial elements in unloading in the xylem region and serve as an important, regulatory step in solute exchange at the symplasm/apoplasm interface.

4.4.3.3 The Involvement of Xylem Parenchyma Cells in Embolism Repairs

Contact cells are also an important factor in a mechanism leading to embolism repairs enabling proper long-distance apoplasmic transport. The role of contact cells in the refilling of cavitated vessels was intensively studied in many species like *Juglans regia* (Améglio et al. 2001, 2002, 2004; Sakr et al. 2003; Decourteix et al. 2006, 2008), *Populus* sp. (Secchi et al. 2011; Secchi and Zwieniecki 2012), *Laurus nobilis* (Salleo et al. 2004, 2009), or in two savanna trees—*Schefflera macrocarpa* and *Caryocar brasiliense* (Bucci et al. 2003).

Although the embolism repair mechanism has not been fully elaborated (Nardini et al. 2011; Secchi and Zwieniecki 2011), the involvement of xylem parenchyma, especially contact cells, in this process is unquestionable (Améglio et al. 2001, 2002; Bucci et al. 2003; Améglio et al. 2004; Salleo et al. 2009; Secchi et al. 2011; Secchi and Zwieniecki 2011, 2012). Firstly, xylem parenchyma cells are a source of sugars and energy. Sucrose, released from stored starch, can directly increase sap osmolarity and xylem pressure (Améglio et al. 2001, 2004) due to its translocation to embolized elements via contact cells (Améglio et al. 2004; Salleo et al. 2009; Secchi and Zwieniecki 2012), although it is unknown as to whether sugar efflux into cavitated conduits is facilitated through diffusion (Améglio et al. 2004) or is an active process (Salleo et al. 2004; Zwieniecki and Holbrook 2009; Secchi and Zwieniecki 2012). Sucrose also acts as a source of energy and indirectly increases sap osmolarity, through ion efflux into apoplasm, via respiration (Secchi et al. 2011; Secchi and Zwieniecki 2012). Regardless of the sucrose translocation pathway and the mechanism of osmotic gradient generation, defoliation in the vegetative season (Améglio et al. 2001) and phloem girdling (Salleo et al. 2004) both lead to reduction of the starch content in xylem parenchyma cells and strongly limit winter embolism repair ability and plant survival (Améglio et al. 2001).

When high sugar concentration is obtained in cavitated elements, water flow from neighboring cells according to the concentration gradient leads to dilution of the gases and complete conduit refilling (Zwieniecki and Holbrook 2009). Because xylem parenchyma cells are in close vicinity to gas-filled tracheary elements, they are regarded as one of the natural sources of water necessary for embolism repair (Améglio et al. 2001, 2002; Sakr et al. 2003; Bucci et al. 2003). Living and metabolically active contact cells are also considered as embolism sensor that detects cavitation and triggers the processes leading to conduit refilling. What is the nature of this signal emitted during cavitation? Possibly it can be connected with either wall vibration after mechanical stimulus (Salleo et al. 2008) and/or changes in osmotic concentrations between parenchyma cells and a neighboring tracheary element after water transport disruption (Zwieniecki and Holbrook 2009; Secchi and Zwieniecki 2011, 2012). It is quite plausible that xylem parenchyma cells, and especially contact cells, are also involved in other steps of the embolism repair mechanism.

4.4.3.4 Defense Mechanisms During Vascular Parasite Infection: Heartwood Formation

Xylem parenchyma cells abutting the vessels also play an important role in plant defense mechanisms against fungal (El Mahjoub et al. 1984; Street et al. 1986; Shi et al. 1992; Benhamou 1995; Cooper et al. 1996; Cooper and Williams 2004) and bacterial (Goodman and White 1981; Hilaire et al. 2001) infections spreading via tracheary elements. Contact cells neighboring infected vessels detect pathogens and trigger cellular defense responses. One of the first visible reactions, also found after

injury (Schmitt and Liese 1990; Sun et al. 2008), is the formation of tyloses leading to partial or complete vessel occlusion (El Mahjoub et al. 1984; Clérivet et al. 2000). Besides tyloses, xylem parenchyma cells can also produce and secrete amorphous substances like gels and gums into the vessel lumen (Street et al. 1986; Shi et al. 1992; Benhamou 1995; Clérivet et al. 2000), which physically restrict pathogen movement within the vascular system (Shi et al. 1992). In addition, contact cells participate in chemical defense mechanisms. Living parenchyma cells abutting infected conductive elements have increased cytoplasmic activity (El Mahjoub et al. 1984; Shi et al. 1992). They produce, accumulate, and secrete into the vessels several materials like lipoidal, phenolic, or terpenoid substances (Shi et al. 1992; Benhamou 1995; Cooper et al. 1996; Clérivet et al. 2000), polysaccharides (Clérivet et al. 2000), elemental sulfur (Cooper et al. 1996; Williams et al. 2002; Cooper and Williams 2004), or cationic peroxidase (Hilaire et al. 2001). Some of these are toxic and can serve as chemical inhibitors restricting pathogen growth and reproduction (Shi et al. 1992; Williams et al. 2002). Moreover, xylem parenchyma cells are involved in callose synthesis and deposition along cell walls and pit membranes neighboring the infected vascular elements (Mueller et al. 1994; Benhamou 1995), as well as in the synthesis of pathogenic-related proteins (Benhamou 1995). They also induce a thickening of secondary walls that decreases pit diameter and limits bacteria spreading via pit membranes into living cells (Hilaire et al. 2001). The few examples mentioned clearly illustrate the significance of living xylem parenchyma cells in physical and chemical defense, assuring the high resistance of plants against various pathogens.

Xylem parenchyma cells perform also a crucial role during the process of heartwood formation (Higuchi et al. 1969; Spicer 2005; Nakaba et al. 2012b). These living cells are involved in vessel occlusions, due to tylose formation or gel secretion (Chattaway 1949) as well as in the production and accumulation of secondary metabolites, which are generally known as heartwood substances (Higuchi et al. 1969; Magel et al. 1991; Hillinger et al. 1996; Taylor et al. 2002; Spicer 2005). These compounds are synthesized *in situ* by parenchyma cells from stored reserves at the sapwood-heartwood boundary (Magel et al. 1991; Yang et al. 2004). Heartwood substances belong mostly to the phenolic compounds (e.g., tannins, terpenes, flavonoids, lignans, and stilbenes) or to lipids (Higuchi et al. 1969; Magel et al. 1991; Hillinger et al. 1996; Taylor et al. 2002). Their accumulation in heartwood leads to wood colorization and enhancement of wood decay resistance (Taylor et al. 2002; Spicer 2005).

4.4.3.5 The Impact of Living Cells on the Growth and Differentiation Processes of the Secondary Xylem

Different types of cells like vessels, tracheids, fiber-tracheids, fibers, and axial and radial parenchymas can be formed and arranged in peculiar and, more usually, in regular spatial patterns in the secondary xylem, with additional variation among the

seed plants. The mechanisms responsible for the growth and differentiation of these various xylem cells, and for formation of specific vascular patterns, remain unknown. Possibly, living xylem parenchyma cells can be involved in these processes by acting as a source of signal molecules, which affect the differentiation of xylem elements (Barnett 1982).

Depending on the morphology and distribution of the vessels in the particular growth rings, two major types of wood can be distinguished in angiosperms: diffuse porous or ring porous (Fig. 4.3). In diffuse porous, wood vessels have approximately similar dimensions and are more or less evenly distributed in growth rings. Conversely, the ring-porous wood dimension of the vessels varies considerably during the vegetative season, with large vessels being produced at the beginning of the season, while narrow ones formed in the second part (IAWA 1989). Developmental studies have clearly demonstrated the impact of living xylem cells on the determination of wood architecture. For example, the occurrence of ring-porous wood in *Gmelina* genus depends on the presence and distribution of living axial parenchyma cells (Chowdhury 1953) that are formed in the beginning of ring growth (initial parenchyma). The lack of initial parenchyma or its scanty distribution results in the diffuse-porous structure of the secondary xylem, whereas abundant initial parenchyma sheathing newly formed vessels leads to the ring-porous structure of the wood (Chowdhury 1953). Such a correlation may suggest that initial parenchyma prolongs the process of vessel formation and influences its radial expansion, resulting in a wider xylem element development of the ring-porous wood. However, the nature of the intercellular signals possibly exchanged between parenchyma and the differentiating xylem elements, as well as their route of transport, are unknown, although the fact that both initial parenchyma cells and differentiating xylem elements are alive suggests the involvement of symplasmic route. This supposition requires further evaluation.

The differentiation processes of xylem elements can be influenced by the plasmodesmata as in the young branches of *Sorbus torminalis* (Lachaud and Mauroussset 1996). In this species, the presence of plasmodesmata in the pit membrane probably maintains symplasmic continuity between differentiating xylem elements and forms a temporary symplasmic domain. It is unknown whether or not plasmodesmata in these pit regions are functional, because no dye-coupling experiments have been done yet. However, it seems highly possible that plasmodesmata may influence wood structure, at least in *Sorbus torminalis*, because symplasmic continuity maintained between young xylem elements probably hampers their differentiation and leads to the formation a homogenous wood (Lachaud and Mauroussset 1996). The development of a homogenous wood structure corresponds well with the general principle that cells, belonging to the same symplasmic domain, differentiate in a similar manner (Ehlers et al. 1999; see Chap. 2). However, the impact of plasmodesmata on the xylem structure appears to be more complex and species dependent due to the fact that the presence of symplasmic communication affects both the growth and the differentiation of xylem cells.

4.5 Future Perspectives

Symplasmic transport in the spatial system of living xylem cells is involved not only in nutrient translocation or substance exchange at the symplasm/apoplasm interface but also in the integration of cell-to-cell communication over long distances in plants. Although our knowledge about the function of living xylem cells and symplasmic transport in wood has recently increased, there are still some questions and problems which need further examination. It cannot be forgotten that transport in seed plants is composed of integrated symplasmic and apoplasmic systems which cooperate to assure proper plant development and function. Thus, the efficiency and velocity of symplasmic transport in wood needs to be elucidated to fully explain transport capacities in woody plants. Although the general axial and radial routes of symplasmic transport in secondary xylem were observed using small molecular tracers, studies of possible macromolecule trafficking between living xylem cells, and their impact on xylem element differentiation and wood structure, are also necessary. Furthermore, symplasmic transport in secondary xylem not only is coordinated at the cellular level, by the mechanisms that act during typical plasmodesmata trafficking, but can also be regulated on the supracellular level. Taking into account the spatial regulation of symplasmic transport in active cambium (Sokołowska and Zagórska-Marek 2012) or the wavy propagation of solutes in the phloem tubes (Thompson and Holbrook 2004), the supracellular coordination of symplasmic transport in wood can be possible and facilitate fast and efficient communication over long distances in trees. Thus, knowledge about the mechanisms of symplasmic transport in wood and the comprehensive role of living xylem cells is indispensable in plant biology, leading to a full understanding of how such long-lived plants as trees can grow and function properly.

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Chapter 5
Symplasmic Transport in Phloem Loading and Unloading

Johannes Liesche and Alexander Schulz

Abstract The coordinated distribution of carbohydrates between different plant organs takes place in the phloem. Understanding how carbohydrates are loaded into and unloaded from this long-distance transport system is essential for our understanding of carbon allocation in plants and the mechanism of phloem transport.

In this chapter, we present the current knowledge on how sugars move from the site of production in the leaf parenchyma towards the phloem and how they exit the phloem in sink organs and move to the sites of consumption or storage. The main focus lies on symplasmic transport through plasmodesmata, which is central to all questions of intercellular carbohydrate transport. Recent investigations in non-model plant species, especially the gymnosperms, provide a more comprehensive picture of phloem loading and unloading processes than ever before, but many questions regarding underlying mechanisms, evolution, pathway capacity, and regulation remain.

Keywords Sucrose • Photoassimilate transport • Carbon allocation • Plasmodesmata • Sucrose transporter

Abbreviations

BSC	Bundle sheath cell
CF	Carboxyfluorescein
CC	Companion cell

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IC	Intermediary cell
PD	Plasmodesmata
SE	Sieve element
SECCC	Companion cell/sieve element complex

5.1 Introduction

The coordinated distribution of carbon throughout the whole-plant body is essential for the plant's growth and development. Sugars, especially sucrose as the main carbohydrate, but also ions, amino acids, and various signaling compounds are transported in the phloem. The highly specialized sieve elements (SEs) accommodate a mass flow of phloem sap, which is always directed from sugar producing regions, the sources, to sugar consuming regions, the sinks. The driving force for phloem transport is the osmotically generated pressure gradient between source and sink (see the Chap. 1 for details on transport in the phloem). Since sugars are the main osmotica in plant cells, the processes leading to a high sugar concentration in the source phloem and their reduction in the sink phloem are therefore of major importance for our understanding of how the phloem operates.

In this chapter we will look at the cellular pathways that sugar molecules pass in order to be loaded into or unloaded from the phloem. Phloem loading, as used here, does not only describe the last step, the transfer of sugars into the phloem SEs from neighboring cells, but does indeed comprise all steps from the site of sugar production to their entering the translocation stream. Similarly, unloading involves all steps from exit from the sieve elements to the site of consumption. Accordingly, the loading process can be divided in two parts: transport along the pre-phloem pathway and uptake into the companion cell/sieve element complex (SECCC). Similarly, unloading can be divided into release from the SECCC and post-phloem transport. In earlier literature, phloem loading and unloading generally referred to only the step of sugar uptake and release from SECCC as the importance of the whole pathway for both processes was not realized.

The focus of this chapter lies on functional data regarding the symplasmic movement of carbohydrates. For a detailed review of the structural data on cell connections in the phloem loading and unloading pathways, see Beebe and Russin (1999) and Schulz (2005).

5.2 Symplasmic Transport in Phloem Loading

Historically, an active pumping mechanism for sugar accumulation in SE was assumed necessary for phloem loading for a long time. The discovery of alternative strategies within the last 20 years led to the current detailed picture of loading strategies in a broad range of plant species.

A mechanism for active sugar accumulation in the phloem was first postulated when a higher concentration of sugars in the leaf phloem compared to the surrounding cells was observed (Phillis and Mason 1933; Roeckl 1949). In several model plants, an active pumping mechanism was later verified for sucrose uptake into the sieve element—companion cell complex (SECCC) from the surrounding apoplasm (Giaquinta 1976; Fondy and Geiger 1977) and sucrose transporters were demonstrated to be responsible for this transmembrane transport step (Riesmeier et al. 1992, 1994; Gahrtz et al. 1994). This phloem loading type, where sucrose is released into the apoplasm and then taken up into the SECCC by sucrose transporters, is referred to as active apoplasmic loading.

An active phloem loading mechanism not involving sucrose transporters was found in *Cucurbita* and various other species of herbaceous plants (Turgeon and Beebe 1991). Instead of secretion of sugars into the apoplasm and uptake into the SECCC, sucrose was postulated to enter via diffusion through the abundant PD at the bundle sheath/companion cell interface (Turgeon and Hepler 1989). To prevent diffusion back into the bundle sheath, sucrose is converted into sugar polymers in the specialized companion cells. The sugar polymers are deemed too large to pass through PD on the bundle sheath side but can pass on into the sieve element to be transported in the phloem (Haritatos et al. 1996; McCaskill and Turgeon 2007). Because of the energy-dependent enzymatic reaction and the entry of sucrose into the SECCC through PD, this loading type is referred to as active symplasmic (Rennie and Turgeon 2009).

Analysis of a wider range of plant species regarding phloem loading led to the discovery of passive symplasmic loading, as first described for the willow tree (Turgeon and Medville 1998). This loading type is characterized by symplasmic continuity along the pre-phloem pathway and the absence of active sugar accumulation in the companion cell/sieve element complex (Turgeon 2010). To move sugars into the phloem, a high overall leaf sugar concentration is maintained that is thought to enable diffusion of sucrose into the phloem. This means that mesophyll concentration would need to be higher than in the source phloem SEs, which is indeed indicated by plasmolysis experiments willow (Turgeon and Medville 1998) and poplar (Russin and Evert 1985) and, indirectly, by the considerably higher leaf sugar content of passive symplasmic loaders compared to active loading species (Fu et al. 2011).

The different mechanisms of the three major phloem loading types are summarized in Fig. 5.1. Some species employ variations of these types as they load and transport large amounts of sugar alcohols in the phloem in addition to sucrose or sugar polymers; others can use two loading strategies in parallel (Rennie and Turgeon 2009).

From the above description, it becomes clear that the symplasmic coupling of leaf cells, especially coupling between the SECCC and the surrounding cells, is a defining feature of phloem loading. In a seminal work, Gamalei determined the abundance of PD between companion cells and bundle sheath cells by electron microscopy in a great number of plant species (Gamalei 1989). Although in many cases, this data, together with the visible specializations of the companion cells, is a

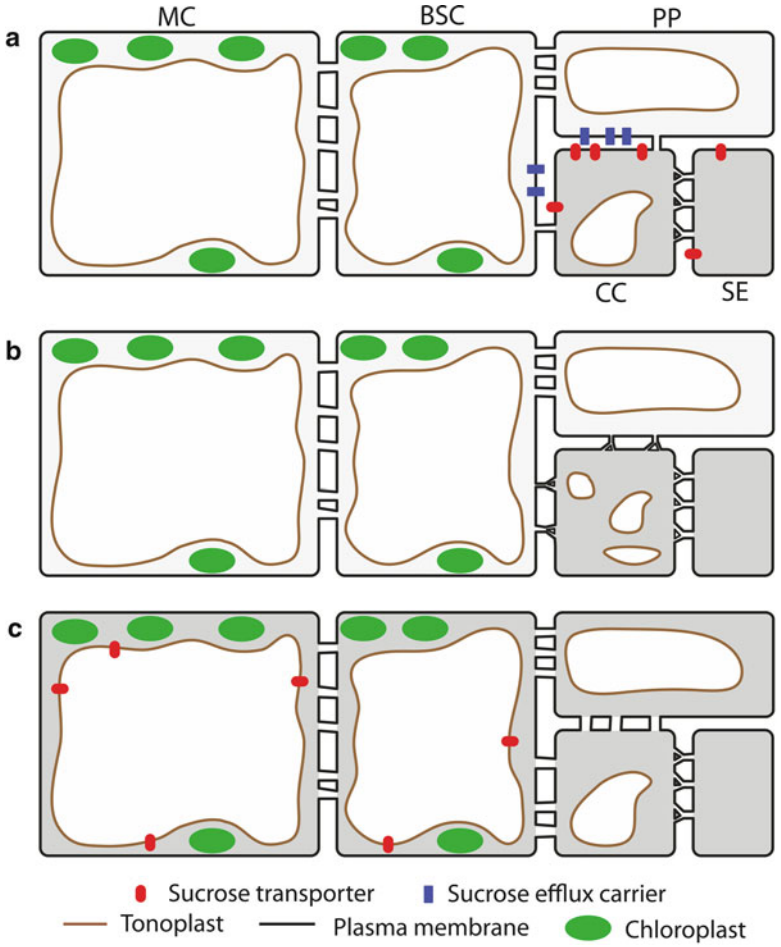

Fig. 5.1 Schematic drawing of phloem loading types. In active apoplasmic loading (**a**), very few plasmodesmata are present between companion cell (CC) and bundle sheath cell (BSC) and phloem parenchyma cell (PP). Sucrose produced in the mesophyll cell (MC) diffuses into BSC and PP but has to be released into the apoplasm by efflux carriers before being taken up into CC and sieve elements (SE) by plasma membrane sucrose transporters. In active symplasmic loading (**b**), sucrose can diffuse into the CC through the abundant plasmodesmata. Sucrose in the CC is converted to sugar oligomers, which, according to the prevalent hypothesis, are too big to diffuse back into BSC or PP but instead enter the SE. In passive symplasmic loading (**c**), sucrose can diffuse along the whole phloem loading pathway from MC to SE following the sugar concentration gradient. Loading could be regulated by import and export of sucrose into/from the vacuole. *Gray* level indicates cytoplasmic sugar concentration

good indication of phloem loading type, PD abundance was shown not to always correspond to loading type but instead be a general feature of leaf anatomy (Turgeon and Medville 2004). While absence of PD is clear evidence for a domain border, their presence does not necessarily indicate free exchange within an individual

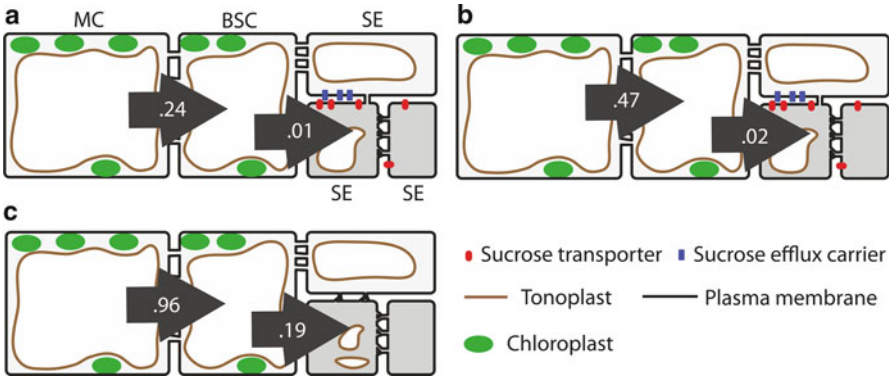

Fig. 5.2 Cell coupling factors in the pre-phloem pathway of the active apoplasmic loading *Vicia faba* (**a**) and *Nicotiana tabacum* (**b**) and the active symplasmic loading *Cucurbita maxima* (**c**). The factor indicates the relative permeability of a cell wall interface for small molecules like sucrose. Based on data from Liesche and Schulz (2012a). All differences are significant. *MC* mesophyll cell, *BSC* bundle sheath cell, *SE* sieve elements

symplasmic domain, as permeability through PD can be dynamic (Schulz 1999; Roberts and Oparka 2003). Recently, the cell wall permeability in the pre-phloem pathway was quantified *in vivo* in several species representing symplasmic and apoplasmic loading types using small fluorescent tracers and live-cell microscopy (Liesche and Schulz 2012a). The results show that in the symplasmic loader *Cucurbita maxima*, the whole pre-phloem pathway is indeed open to diffusion, whereas in the apoplasmic loaders *Nicotiana tabacum* and *Vicia faba*, the SECCC is isolated from the bundle sheath cells (Fig. 5.2).

In the following paragraphs, relevant aspects and open questions of symplasmic transport associated with the different loading types will be discussed.

5.2.1 Is the Phloem Symplasmically Isolated in Apoplasmic Loaders?

In our current understanding of apoplasmic phloem loading, sucrose is released into the cell wall space in proximity to the SECCC of minor veins (Chen et al. 2012). It is then pumped into the SECCC by sucrose transporters located in the plasma membrane of the companion cell and/or sieve element (Stadler et al. 1995; Kühn et al. 1997). This mechanism is thought to enable the plants to keep their whole-leaf sugar content low while still reaching the high concentration necessary to establish the source–sink gradient that drives phloem transport (Turgeon 2010). As a precondition for this mechanism, the SECCC has to be symplasmically isolated from the surrounding cells to prevent backflow of sucrose, which would compromise the pumping process.

All apoplasmic loaders feature at least a few PD in the cell wall between CC and BSC, even those that have been classified "closed" by Gamalei (1989), as was shown, for example, for *Sonchus* (Fisher 1991), *Linaria* (Turgeon et al. 1993), and *Vicia* (Gunning et al. 1974; Delrot and Bonnemain 1981). Microinjection experiment with fluorescent tracers showed no movement out of the SECCC in minor veins of tobacco (Itaya et al. 2002) and barley (Botha and Cross 1997). Similarly, the *in vivo* quantification of cell coupling between bundle sheath and companion cells in the apoplasmic loaders *Nicotiana tabacum* and *Vicia faba* showed that virtually no tracer could pass this interface (Fig. 5.2), indicating that the traffic through the PDs that are present might be restricted. Nevertheless, slight but significant differences were apparent between the bean plants that feature fewer PD at the BSC–CC interface than tobacco plants, which itself has only a low number of PD (Liesche and Schulz 2012a). It remains to be seen if apoplasmic loading plants that do possess a high number of PD between BSC and CC, like *Vinca major* (Rennie and Turgeon 2009), show markedly higher permeability or if the PD are blocked.

Electron microscopy has shown in one case, *Moricandia arvensis*, that PD between CC and vascular parenchyma appear occluded and in lack of a desmotubule (Beebe and Evert 1992). In all other cases, no difference in PD structure compared to PD between mesophyll cells is visible (Turgeon 2006).

From an evolutionary perspective, it is puzzling that at least some PDs are present in apoplasmic loading species, even though they are potentially counterproductive for the loading process. More important, these PDs are likely to be the entrance door for plant viruses to the long-distance transport system, leading to systemic spread of virus particles which often results in the death of the host plant (Leisner and Turgeon 1993).

How exactly the entrance of viruses into the phloem happens is not clear, but the mechanism appears to differ from the intercellular movement of viruses between mesophyll cells. For example, it was shown that tobacco mosaic virus can move between mesophyll cells without coat protein, but not into SECCC (Ding et al. 1996), and the movement protein of cucumber mosaic virus expressed in tobacco minor vein CCs can leave the SECCC even though fluorescein and dimeric GFP cannot (Itaya et al. 2002).

Even though entry into SECCC is the critical step for systemic infection, as seen by the high accumulation of virus particles in phloem parenchyma before the spread, the time of this process appears to be independent of the number of PD as demonstrated in infection experiments with various viruses on Solanaceae and Fabaceae species (Ding et al. 1998). This suggests that viruses are able to induce an effective symplasmic pathway into the phloem, even though, at this stage, an alternative apoplasmic mechanism cannot be entirely ruled out.

Having a potentially negative effect on the competitiveness of a plant, the PDs must fulfill an essential function in a healthy plant, or otherwise, evolutionary pressure should have led to their elimination. Complete elimination of PD connections can, for example, be seen in guard cell development. They do not offer the potential to serve in phloem unloading as differentiated source organs never turn back to being sink (Turgeon 1986), but there is the possibility that the PDs enable passive

symplasmic loading (Schulz 2005). Growth of Arabidopsis plants expressing a nonfunctional AtSUC2 was stunted but not completely abolished, showing that sucrose export works to a limited extent without active loading into the SECCC (Srivastava et al. 2008). The same could be observed in maize SUT1 mutants (Slewinski et al. 2009). Although these mutants benefit from the capacity for phloem loading, it is unlikely that the PDs are present for this purpose. More likely is a role in signaling. It was proposed that there is constant communication between CC and mesophyll to fine-tune photosynthesis and sucrose export (Lucas et al. 1996). There are indeed strong arguments for a sink-dependent regulation of photosynthesis (Paul and Foyer 2001), which would require communication between SECCC and surrounding cells.

5.2.2 The Mechanism of Polymer Trapping in Active Symplasmic Loaders

In active symplasmic phloem loading, the interface between SECCC and the neighboring cells of the upstream pre-phloem pathway is fundamental to the loading mechanism, just like in apoplasmic loaders. Here, too, the concentration of sugars is higher in the phloem than in the rest of the leaf and therefore requires loading against an overall concentration gradient. This gradient has to be maintained, even though the SECCC is not symplasmically isolated, but well coupled with the surrounding tissue (Voitsekhovskaja et al. 2006). To achieve this, enzymes that polymerize sucrose into raffinose and stachyose are active specifically in the companion cells, which are called intermediary cells (IC) (Holthaus and Schmitz 1991; Turgeon et al. 1993; McCaskill and Turgeon 2007). As the sucrose concentration is thereby constantly reduced, it can be replenished by diffusion from mesophyll to phloem along the concentration gradient. It is clear that this mechanism requires anatomical specialization of the IC that is highly interesting in terms of symplasmic transport. PDs towards the upstream neighbor cells have to accommodate sucrose movement but restrict outflow of higher molecular weight sugars. The PDs towards the SE have to accommodate passage of high amounts of the sugar polymers as they are the transport sugar. Indeed, all active symplasmic loaders can be identified by the presence of IC with their unique anatomy (Fig. 5.3).

The PDs between ICs and BSC are very abundant, concentrated in fields, and highly branched and have unique neck region on the intermediary cell side, consistently described as narrow (Fisher 1986; Turgeon et al. 1993; Volk et al. 1996). PD neck regions are generally thought to be the decisive part for regulation of the rate of intercellular transport (Schulz 1995).

Besides the circumstantial evidence from structural analysis, it is difficult to prove the sucrose selectivity of these PD. It was shown that the hydrodynamic radius is decisive for traffic through PD for small molecules (Terry and Robards 1987). The hydrodynamic radius of sucrose in cytosol was estimated to be 0.44 nm (Sowinski et al. 2008) and that of raffinose is only about 0.1 nm larger (Schultz and

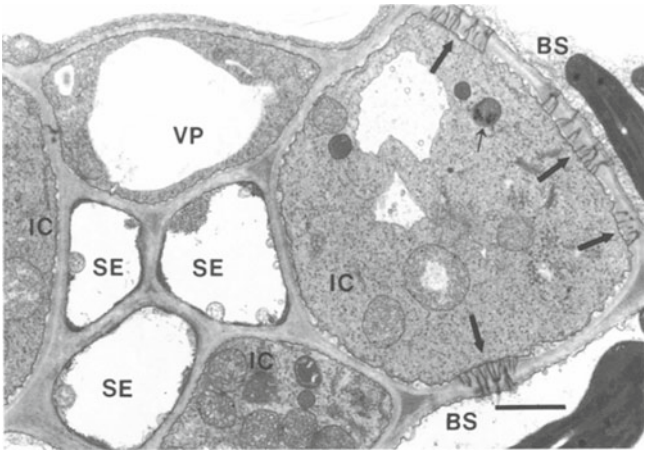

Fig. 5.3 Electron micrograph of a minor vein of the active symplasmic loader *Alonsoa warscewiczii* showing a typical intermediary cells (IC). IC are connected to bundle sheath cells (BS) via abundant extensively branched plasmodesmata (*arrows*). *SE* sieve element, *VP* vascular parenchyma. Scale bar 2 µm (reprinted from Turgeon et al. (1993), with kind permission from Springer Science+Business Media)

Solomon 1961). Terry and Robards (1987) injected dyes of different hydrodynamic radii into the tip of *Abutilon* nectary trichomes and monitored the spread of fluorescence along the line of cells. Even though the PDs analyzed in this study clearly have a wider cytoplasmic channel radius compared to the BSC/IC PD, the results show that, in general, size discrimination is only effective when the radius of the cytoplasmic channel is little larger than the smaller molecule [see Fig. 8 in Terry and Robards (1987)]. To prevent passage of raffinose, the channel would need to be considerably below 1 nm in diameter, considerably smaller than normal PD, whose cytoplasmic channels are usually estimated to be around 3–4 nm in diameter (Ding et al. 1992; Fisher 1999).

Nevertheless, it is clear that the PDs are responsible for the filtering effect, as raffinose biosynthesis without structural specialization, which was simulated by expressing raffinose synthase in tobacco CC, does not result in efficient phloem transport (Hannah et al. 2006). Furthermore, recent data on the plasmodesmata-mediated cell wall permeability in the active symplasmic loader *Cucurbita maxima* allowed the calculation of conductive area that is necessary to accommodate efficient sucrose movement. Theoretical optimization indicates that the requirements can be met, even if the diameter of the cytosolic channels inside the PD is below 1 nm, i.e., small enough for efficient size discrimination (Liesche and Schulz, unpublished data). If size discrimination is the decisive factor for the polymer trap or if other, as yet undiscovered, components are responsible for the preference of sucrose transport remains to be determined.

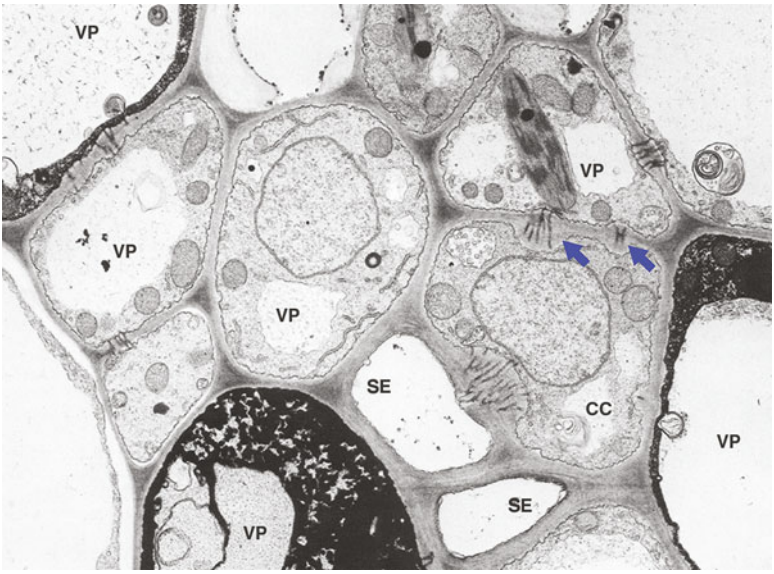

Fig. 5.4 Electron micrograph of a minor vein of the passive symplasmic loader *Populus deltoides*. The companion cell (CC) is connected to the vascular parenchyma (VP) via conspicuous plasmodesmata (*arrows*). As in all angiosperms, CCs are connected to sieve elements (SE) by complex plasmodesmata aggregates (reprinted from Russin and Evert (1985), with kind permission from The Botanical Society of America)

5.2.3 The Mechanism of Passive Symplasmic Phloem Loading

Efficient carbon allocation demands a tight but flexible control of carbon export to meet sink demand under a wide range of environmental conditions (Smith and Stitt 2007). In active apoplasmic and active symplasmic loaders, the sucrose transporters and the sucrose-oligomerizing enzymes are thought to be the respective main control points (Ayre 2011) to adapt export rates to, for example, changed nitrogen availability. In symplasmic loaders, where phloem and mesophyll are directly coupled, export from source organs could be modulated by control of sucrose levels in the cytosol. A larger concentration potential along the pre-phloem pathway would lead to higher diffusion rates and thereby increased phloem loading.

Even though the loading type is termed passive symplasmic loading, there are indications that active transport processes are involved in the control of the cytosolic sucrose concentration in the different cells. In poplar, a vacuolar sucrose transporter was shown to have a strong effect on the phloem loading capability (Payyavula et al. 2011). Also in rice, where the phloem loading type has not yet been conclusively determined, a concept with a vacuolar sucrose transporter essential for efficient

export has been proposed (Eom et al. 2012). The coordinated pumping of sucrose from the cytosol into the vacuole and release from vacuole to cytosol by sucrose transporters could enable direct control of phloem loading.

Alternatively, phloem loading could also be controlled by dynamic regulation of plasmodesmata, thereby changing the transport capacity of the symplasmic pathway, which has been demonstrated in phloem unloading in pea roots (Schulz 1995) but has not been shown in relation to phloem loading. Nevertheless, the conspicuous PDs seen, for example, in *Populus deltoides* (Fig. 5.4) (Russin and Evert 1985), are indicative of the potential for permeability regulation at the neck region (Schulz 1999). Sucrose conversion to and from starch in the plastids could also help to balance cytosolic sucrose concentration, although starch accumulation follows strictly diurnal patterns and offers therefore no option for short-term adaptation.

Another fundamental question regarding the mechanism of passive symplasmic phloem loading is the interaction of water moving outwards from the xylem towards the stomata and sugar molecules moving inwards towards the phloem. It is unclear to what extent the high sugar concentration in the mesophyll cells leads to osmotic water uptake and the buildup of osmotic pressure. The buildup of osmotic pressure would virtually extent the phloem into the mesophyll with bulk flow not starting in the SECCC, but already in mesophyll cells. Whether sugar moves in the leaf by bulk flow or diffusion has important implications for the understanding of not only phloem loading but also leaf functional architecture.

5.2.4 Phloem Loading in Gymnosperms

For a long time, research on phloem transport has been focused on angiosperms. Important questions of how the phloem evolved and how it is implemented in plants with vastly different architectures require analysis of a broader range of plant groups.

Gymnosperms, which are today mostly represented by the conifer species, include trees with the longest source–sink distance of any plant on earth. Despite their enormous size and numerous other anatomical specialties (Schulz 1990, 1992), the same basic mechanism of phloem transport seems to be employed as in angiosperm trees and herbaceous plants (Jensen et al. 2012). Recent work on phloem loading in gymnosperms helps to understand how this mechanism is implemented in this group of plants.

Gymnosperm leaves, in most cases needle-shaped, have a very complex architecture (Huber 1947). The pre-phloem pathway consists of four different cell types and as much as nine cells in between a mesophyll cell and an SE (Fig. 5.5, Canny 1993; Liesche et al. 2011b; Liesche and Schulz 2012a). It features an extensive transfusion tissue, which is specific for gymnosperms and includes dead transfusion tracheids and living transfusion parenchyma cells (Liesche et al. 2011b).

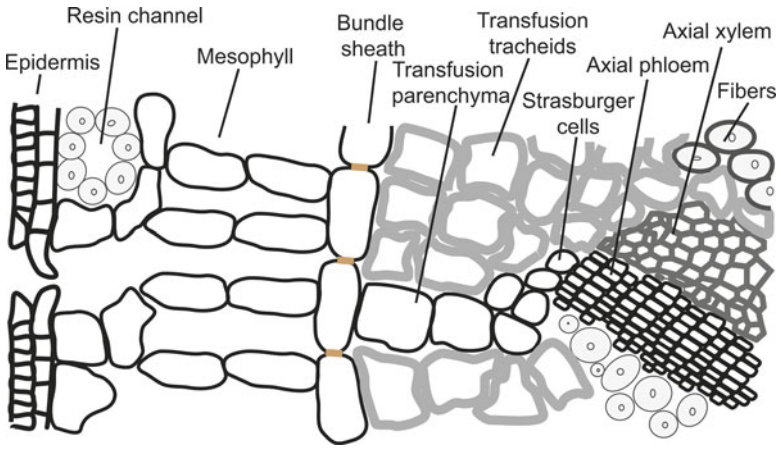

Fig. 5.5 Schematic drawing of the pre-phloem pathway in the pine needle. The endodermis-like bundle sheath with Casparian strip and the transfusion tissue consisting of dead water-filled tracheids and living parenchyma cells are features that can be found in all gymnosperm leaves. The Strasburger cells of gymnosperms have similar function to the angiosperm companion cells

The transfusion parenchyma and the Strasburger cells, which are comparable in function to the CC of angiosperms, form a bridge of living cells between the bundle sheath cells and the phloem sieve elements (Fig. 5.5).

Symplasmic coupling of the pre-phloem pathway in mature conifer needles is indicated by the presence of PD at all cell wall interfaces along the pathway as seen by transmission electron microscopy (Blechschmidt-Schneider et al. 1997; Liesche et al. 2011b). The structural data was complemented by *in vivo* measurement of cell coupling in the pine tree *Pinus sylvestris*, where the cell coupling factor for all interfaces was found to be similar to that of the well-coupled *N. tabacum* mesophyll cells (Liesche and Schulz 2012a). Leaf sugar concentration, as indicated by the leaf sap osmolality, was also found to be high, slightly higher than the average for the angiosperm trees with passive symplasmic loading (Liesche and Schulz 2012a). This indicates a passive symplasmic loading type.

Further evidence was provided already by leaf-disc assays, a test for the involvement of active processes in phloem loading. Usually, a leaf cutout is incubated in radioactively labeled sucrose, resulting in accumulation of signal in the leaf veins in active loading species, whereas symplasmic loading species show a homogeneous distribution of radioactivity (Turgeon and Medville 1998). Conifer needles are not compatible with this test because of their anatomy, but when allowed to assimilate $^{14}CO_2$, which is then built into ^{14}C-sucrose, the distribution can be detected when cross sections are exposed to photographic film. First experiments with *Pinus mugo* needles, whose structure is similar to that of *P. sylvestris* needles, showed a homogeneous signal distribution in the living cells (Fig. 5.6).

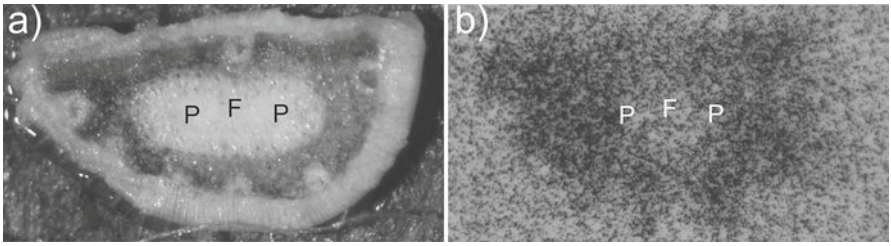

Fig. 5.6 Distribution of radioactive assimilates in a *Pinus mugo* needle. The needle was allowed to assimilate $^{14}CO_2$ for 1 h, freeze-dried and sectioned. The cross section (**a**) was exposed to photographic film (**b**) for 20 h. Signal (*dark* area) is visible in all living cells of the needle, including the outer axial phloem (P), but not the dead fibers (F) in the center. See Fig. 5.5 for an illustration of needle anatomy

Combined, these results indicate that in *P. sylvestris* the same principle of phloem loading is at work as described as passive symplasmic loading in woody angiosperms. The results further corroborate the assumption that the phloem loading type of a plant is related to its life form (Turgeon 2010; Davidson et al. 2011). *P. sylvestris*, like the majority of angiosperm tree species, does not seem to rely on active loading, which appears only to be necessary for herbaceous plants with their rapid-growth strategy.

The leaf-vein anatomy of the source organs is conserved across gymnosperm species. Key features, like the endodermis with Casparian strip and the transfusion tissue, are shared not only between the needle-bearing trees but also distantly related species, like the tropical tree *Gnetum gnemon* or of the desert-growing shrub *Welwitschia mirabilis* (Liesche et al. 2011b).

Preliminary results from photobleaching experiments with *G. gnemon* leaves show a high symplasmic coupling between mesophyll cells and sieve elements, similar to *P. sylvestris*. Photobleaching of fluorescent tracers in the phloem of the broad-leaved *G. gnemon* led to significant reduction in fluorescence in the mesophyll cells due to the relocation of tracer molecules. The same effect could be observed in the symplasmic loader *Populus trichocarpa*, but not in the apoplasmic loader *N. tabacum* (Liesche, unpublished data).

The leaf sap osmolality in *G. gnemon* was found to be even slightly higher than in *P. sylvestris* (Liesche and Schulz 2012a). High total leaf sugar, a criteria for passive symplasmic loading, was also found in the deciduous conifer *Larix deciduas* and the evergreen conifers *Abies alba*, *Picea abies*, and *P. sylvestris*, as indicated by levels of nonstructural carbohydrates that were as high or higher as in six deciduous angiosperm trees growing under similar conditions (Hoch et al. 2003).

The broad-leaved gymnosperm *Ginkgo biloba* has been analyzed by leaf-disc assay, the test for active facilitation of loading described above, and found not to accumulate radioactive sucrose in leaf veins, again indicating a passive loading type (van Bel et al. 1994; Blechschmidt-Schneider et al. 1997).

The results point towards utilization of a passive symplasmic phloem loading mechanism by all gymnosperms.

5.2.5 *The Evolution of Phloem Loading*

The analysis of loading type distributions within the angiosperms has not yielded conclusive results as to which mechanism is the most ancestral one. In general, abundant PDs at the BSC–CC interface seems to be ancient and the reduction in PD number at this interface derived, but the passive symplasmic loading of *Salix* seems derived and active symplasmic loaders can be found at all stages (Turgeon et al. 2001). Indeed, one of the most ancestral angiosperm species, *Amborella trichocarpa*, uses the polymer trap mechanism for phloem loading (Turgeon and Medville 2011).

In order to understand the evolution of phloem loading, it is therefore necessary to analyze more ancestral plants, namely, the pteridophytes and the bryophytes, which feature a phloem precursor tissue. The only data available so far from these groups of plants concern the presence of sucrose transporters. The fully sequenced *Selaginella moellendorffii* and *Physcomitrella patens* both feature sucrose transporters of the type 2 and type 4 families but not of the type 1 family that is responsible for active apoplasmic phloem loading (Kühn and Grof 2010). The function of type 2 and 4 transporters is not well understood, but at least some members seem to play an important role in phloem loading (Reinders et al. 2012).

So far, it can be concluded from the structural and molecular data that active apoplasmic phloem loading is derived and occurs only in angiosperm species. While a development of phloem loading originating from a passive symplasmic mechanism seems most plausible, the origin of the process remains unexplained.

5.3 Symplasmic Transport in Phloem Unloading

Phloem unloading, the exit of nutrients from the SECCC and the post-phloem movement towards the site of consumption and storage, is at least as versatile as their phloem loading path. The different anatomies of plant organs that depend on the supply of carbohydrates via the phloem require adaptation of the phloem unloading mechanism. In addition, phloem unloading has to match the developmental stage of the sink organ.

As in phloem loading, the abundance of PD along the various interfaces, as determined by TEM, is an important characteristic of unloading pathway (Fisher and Oparka 1996). The use of radioactively labeled sugars provided the first functional data on the question whether unloading follows a symplasmic or apoplasmic pathway (Dick and Rees 1975). Dick and Rees (1975) saw no influence of sugar solutions on the transport of radioactive photoassimilates towards the tip of *Pisum sativum* roots, concluding that phloem unloading is symplasmic. A big

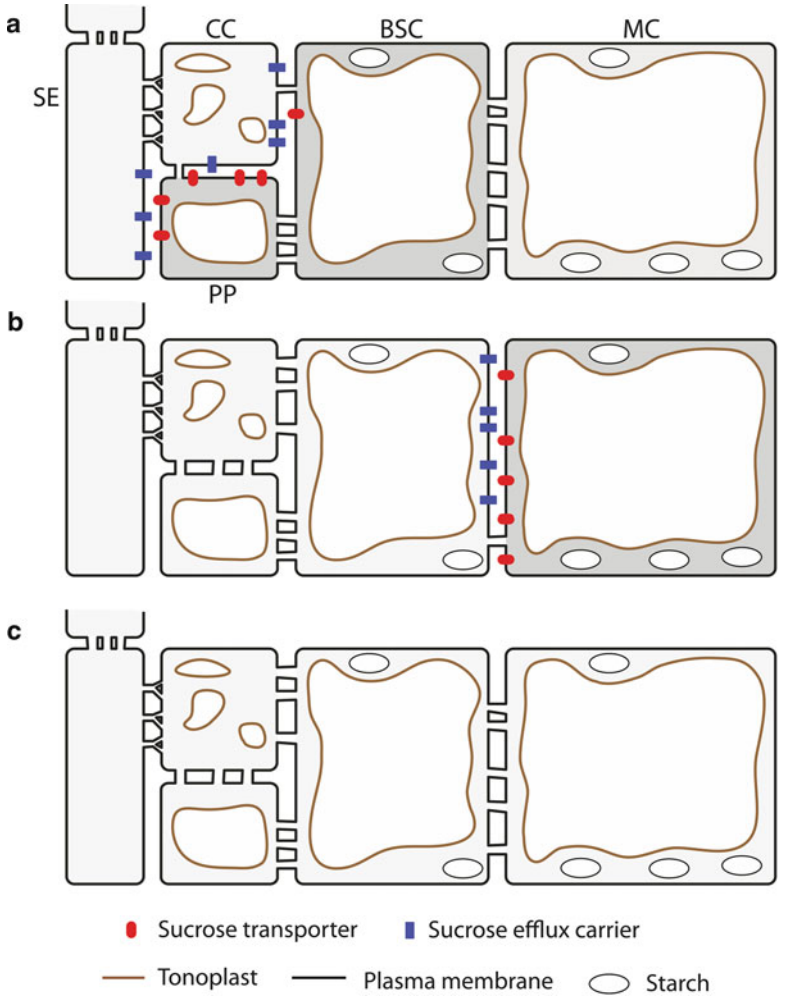

Fig. 5.7 Schematic drawing of phloem unloading types. Apoplasmic unloading involves a symplasmic barrier either around SECCC (**a**) or at an interface further along the post-phloem pathway (**b**). In some cases sucrose cleaving enzymes located in the cell wall digest sucrose, and monosaccharides are subsequently taken up into the cells. Apoplasmic unloading enables higher sugar concentrations in the sink cells compared to the sink phloem. In symplasmic unloading (**c**), sugar diffuses from the phloem to the sites of consumption or storage through plasmodesmata along the concentration gradient. *Gray* value indicates cytoplasmic sugar concentration. *CC* companion cell, *BSC* bundle sheath cell, *PP* phloem parenchyma cell, *MC* mesophyll cell, *SE* sieve elements

advancement for the identification of symplasmic domains in sink tissues was the introduction of fluorescent tracers that could be loaded in the leaf and then move with the phloem and along the post-phloem pathway as far as symplasmic coupling exists (Oparka et al. 1994, 1995; Roberts et al. 1997). A similar approach could later

be realized with GFP expressed specifically in companion cells (Imlau et al. 1999). These plants have been instrumental for the determination of SEL in sink tissues (Imlau et al. 1999; Oparka et al. 1999; Wright et al. 2003). Recently, the technological development of NMR has enabled to resolve distribution of sugar and other compounds in seeds (Melkus et al. 2011) and storage organs (Jahnke et al. 2009).

In general, phloem unloading pathways are categorized in three types that differ in the presence of an apoplasmic step and the position of it (Fig. 5.7). Whereas active apoplasmic and passive symplasmic unloading are analogous to the respective phloem loading types, there is no equivalent to the active symplasmic loading. It is also important to note that active apoplasmic unloading does not necessarily involve sucrose transporters, like in phloem loading. Instead, sucrose can be cleaved into glucose and fructose by sucrose synthase or, after export to the apoplasm, invertase, and the monosaccharides are then taken up into the surrounding cells by respective transporters. The implementation of the different types varies with the specific conditions in each sink organs, which will be discussed in detail in the following section.

5.3.1 Phloem Unloading in Different Sink Organs

5.3.1.1 The Root

Vegetative root growth requires supply of photoassimilates mainly to the subapical meristematic region and the elongation zone just above. In the elongation zone, new sieve elements differentiate, but companion cells might be lacking (Eleftheriou 1990; Schulz 1994). In this so-called protophloem, PDs between sieve elements and phloem parenchyma cells are abundant as seen on electron micrographs in *Pisum sativum* (Schulz 1995), *Zea mays* (Warmbrodt 1985b; Bret-Harte and Silk 1994), *Hordeum vulgare* (Warmbrodt 1985a), and *Allium cepa* (Ma and Peterson 2001), indicating a capacity for symplasmic unloading.

In the roots of an *Arabidopsis thaliana* seedling, phloem unloading in a sink organ could be visualized for the first time *in vivo* (Oparka et al. 1994). The symplasmic tracer carboxyfluorescein (CF) was applied to one cotyledon, where it entered the phloem. The following transport in, and unloading from, the phloem was followed using a confocal microscope. It was shown that both SE unloading and post-phloem transport are symplasmic and that phloem unloading is restricted to the growth zone at the root tip, with no tracer leaving the vasculature at any other region along the root (Oparka et al. 1994). Nevertheless, PDs are also present between SECCC and adjacent cells at the other regions, since unloading can be induced along the whole root by application of metabolic inhibitors (Wright and Oparka 1997). The tracer results are validated by the calculation of the effective transport rate across the SE plasma membrane. In the most apical segments of growing pea roots, this rate is 20–50-fold the amount sucrose transporters could possibly effectuate (Fig. 5.8).

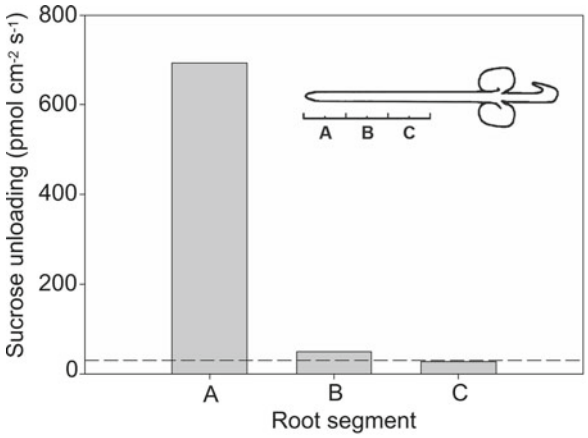

Fig. 5.8 Phloem unloading in the pea root tip cannot be apoplasmic. Calculations based on the combined sieve element surface and current estimates of sucrose transport capacity would allow for unloading of sucrose at a rate of about 30 pmol cm^{-2} s^{-1} (*dashed line*). This flux is more than 20 times too low to explain the observed sucrose unloading in the apical region (A). Only in region C, which is part of the transport phloem, apoplasmic unloading is feasible. Based on data from Schulz (1998) and Peiter and Schubert (2003)

Similar experiments, using GFP instead of CF, which is expressed in companion cells and transported in the phloem demonstrated that the size exclusion limit of PD in the Arabidopsis root tip and other sink organs is higher than in other tissues (Imlau et al. 1999). The 27 kDa GFP cannot pass regular PD, for example, in the leaf mesophyll cells but is unloaded from the phloem in the same pattern as seen with CF.

A direct proof for symplasmic unloading in roots was provided in *Pisum sativum* seedlings, where radioactive sucrose, which was applied to the cotyledons, stopped to accumulate in the root tip after plasmolysis of the root cells (Schulz 1994). In addition, it was shown that the amount of unloaded sucrose is directly correlated with PD diameter between cortex cells (Schulz 1995).

Even though analysis of phloem unloading in vegetative growing roots has been limited to a relatively small number of plant species, the strictly symplasmic pathway can be assumed for all plants as anatomy and growth pattern are sufficiently similar.

In contrast to vegetative growing roots, unloading in storage root has been reported to involve an apoplasmic step. In the root of sugar beet (*Beta vulgaris*), which accumulates sucrose in the storage parenchyma cells, preliminary results indicate that PD frequency between CC and storage parenchyma is extremely low with 0.09 PD μm^{-1} interface and even lower between storage parenchyma cells (0.005 PD μm^{-1}) (Mierzwa and Evert 1984). Sucrose-uptake experiment with sugar beet root tissue discs revealed sucrose-transporter-mediated uptake from the apoplasm (Wyse 1979; Lemoine et al. 1988). However, participation of a symplasmic

unloading pathway cannot be ruled out. As sucrose accumulation was not sensitive to external application of the sucrose-transporter inhibitor PCMBS, the possibility of symplasmic transport plus active uptake into vacuoles was raised (Giaquinta 1979). It should be noted that sugar beet anatomy is complex, and sucrose is stored not only in root but also in hypocotyl and lower stem (Mahn et al. 2002).

In the storage root of carrot (*Daucus carota*), sucrose is accumulated in the main storage phase, and concentration in cells surrounding the phloem is high, reaching 200 mM (Korolev et al. 2000). A sink-specific sucrose transporter was identified (Shakya and Sturm 1998), indicative for apoplasmic unloading.

The limited data suggests that sucrose-storing roots use apoplasmic phloem unloading. It would be interesting to see if the same is true for starch-storing storage roots, like sweet potato.

5.3.1.2 The Shoot

Nutrients are needed in the stem for apex and elongation growth and in some species also for storage and secondary growth. The difference of function of these different sink tissues is reflected in different phloem unloading mechanisms.

Unloading at the vegetative apex of the shoot was found to be more complex than unloading at the root apex, even though the anatomical situation is similar with the first step of unloading being the exit of photoassimilates from protophloem SEs. Distinct symplasmic domains were identified by restricted tracer movement between certain cell layers in *Egeria densa* (Erwee and Goodwin 1985) and *Solanum tuberosum* (van der Schoot and Lucas 1995). The unloading pathway was elucidated in more detail in Arabidopsis by monitoring the unloading of the fluorescent tracer HPTS that was applied to source leaves (Gisel et al. 1999). Here, a symplasmic unloading from the protophloem sieve tubes was observed, but subsequent tracer movement was limited to the outermost cell layer. Symplasmic coupling between different cell layers in the shoot apex is essential for the development of meristematic cells into different organs as it defines the distribution of non-cell autonomous signals, such as the KNOTTED1 protein (Lucas et al. 1993; Rinne and van der Schoot 1998; Gisel et al. 1999; Gisel et al. 2002; Kim et al. 2003).

In the elongating internodes of the shoot, where the phloem is fully differentiated, the SECCC appears to be isolated in angiosperms. Extensive studies have been conducted with *Phaseolus vulgaris*, showing that CF does not exit the SECCC and that radioactive sucrose is unloaded even though the cells of the post-phloem pathway are plasmolyzed (Minchin and Thorpe 1984; Wood et al. 1998). High sucrose concentration in the apoplasm is another indicator for secretion in the cell wall space before uptake by the surrounding cells (Patrick and Turvey 1981; Minchin et al. 1984). Apoplasmic sucrose levels rise when sucrose uptake is inhibited by PCMBS (Minchin and Thorpe 1984) and decreases when photosynthetic activity is low (Patrick and Turvey 1981). Phloem unloading in elongating internodes of the monocot *Sorghum* seems to happen in the same way as in dicots, with much higher sucrose levels in the apoplasm compared to the symplasm (Tarpley and Vietor 2007).

Nevertheless, even in *Phaseolus*, unloading might become symplasmic in response to a change in environmental conditions (Hayes et al. 1987), and data from a wider range of plant species is necessary to identify a common principle of phloem unloading in elongating internodes.

Increasing symplasmic isolation of the SECCC was shown to be concomitant with the maturation of phloem in the stem of *Lupinus luteus* by dye-coupling experiments (van Bel and van Rijen 1994). The low frequency of PD between SECCC and surrounding cells in the stem was shown in many herbaceous angiosperms (Kempers et al. 1998). Nevertheless, considerable amounts of sugars leak out of the transport phloem, either by diffusion through PD or facilitated by efflux carriers (Thorpe and Minchin 1996; Liesche and Minchin, unpublished data). Sucrose transporters are necessary to reload sugar, as demonstrated by significantly increased phloem transit times in AtSUC2 knockout Arabidopsis (Gould et al. 2012) and increased sucrose efflux in stem parts that are treated with sucrose transport inhibitors (Minchin and Thorpe 1984). The transporter-mediated retrieval along the transport phloem was shown to dynamically adjust to sink strength (Eisenbarth and Weig 2005).

Symplasmic isolation of the SECCC in the mature stem is unlikely for plants with secondary growth. At least for a large number of gymnosperms, PD abundance at the interface between SEs, Strasburger cells, and phloem parenchyma cells indicates a symplasmic phloem unloading pathway (den Outer 1967; Sauter et al. 1976).

Two stem storage organs have received considerable attention regarding their mechanism of phloem unloading, the storage parenchyma in the internodes of sugarcane (*Saccharum* species) and the potato (*Solanum tuberosum*) tuber. In case of sugarcane, a symplasmic post-phloem pathway was proposed as cell walls outside the vasculature become lignified and suberized with maturation preventing diffusion of tracers in the apoplasm (Jacobsen et al. 1992; Walsh et al. 2005), which is supported by the presence of PD between CC and bundle sheath cells, neighboring bundle sheath cells as well as bundle sheath cells and storage parenchyma (Welbaum et al. 1992; Botha et al. 2004). An apoplasmic step might still be involved as Botha and coworkers detected large callose deposits in the neck region of PD in the cell walls of storage parenchyma cells (Botha et al. 2004). Indeed, with the exceptionally high concentrations of sucrose measured in the storage parenchyma of sugar cane, it would be surprising to find a completely symplasmic phloem unloading mechanism, as it would require even higher concentrations in the source phloem, although, as in the sugar beet storage tissue, tonoplast-transporter-facilitated storage in the vacuole has to be taken into account as well (Getz et al. 1991).

In the potato tuber, phloem unloading switches from apoplasmic to symplasmic during development. In the elongating stolon, CF cannot exit the SECCC (Hancock et al. 2008). In contrast, in the swelling stolon (the actual tuberization), tracer diffuses into the storage cells (Viola et al. 2001) through the abundant PD (Oparka 1986). Interestingly, the vasculature of the dormant tuber seems isolated but becomes connected again with budbreak in the vicinity of the growing bud (Hancock et al. 2008).

5.3.1.3 The Sink Leaf

Before the sink/source transition, developing leaves of dicotyledonous angiosperms are supplied with phloem-delivered photoassimilates that are unloaded from the major veins (Roberts et al. 1997; Wright et al. 2003; Turgeon 2006). All investigations point to a completely symplasmic mechanism of phloem unloading as shown by high abundance of PD at all interfaces of the post-phloem pathway (Ding et al. 1988; Roberts et al. 2001), GFP and CF diffusing out of CC into neighboring cells (Roberts et al. 1997; Oparka et al. 1999), apoplasm fluid analysis, and test of sucrose-transporter activity (Schmalstig and Geiger 1985; Turgeon 1987; Lemoine et al. 1992).

In monocots, the situation seems to be similar, indicated by high PD frequencies and diffusion of CF out of the SEs in barley leaf (Haupt et al. 2001). Earlier reports proposed an apoplasmic mechanism for phloem unloading in leaves of maize (Evert and Russin 1993) and barley (Evert et al. 1996), but the observed scarcity of PD was probably due to analysis of leaves that already underwent transition to source (Haupt et al. 2001).

5.3.1.4 Flower, Seed, and Fruit

Unloading in the flower of Arabidopsis has been studied in detail recently and a link between symplasmic coupling in the post-phloem pathway and the complex pattern of flower development established (Gisel et al. 1999; Werner et al. 2011). SECCC unloading switches from symplasmic to apoplasmic with the maturation of ovules but is symplasmic again after anthesis in the open flower (Werner et al. 2011). The post-phloem pathway is divided into distinct symplasmic domains that change according to the different developmental stages.

Unloading from *Digitalis purpurea* nectaries is apoplasmic, which is indicated by the very low frequency of PD between SECCC and surrounding cells, together with high apoplasmic sugar concentration. The authors argue that this is a general feature of plant nectaries (Gaffal et al. 2007).

The extensive research on seed development, especially in legumes and cereals, led to the conclusion that developing seeds are symplasmically isolated from the parent plant (Patrick and Offler 2001). PD were shown to be virtually absent from this maternal-filial boundary (Felker and Shannon 1980; Offler and Patrick 1993), and CF and other symplasmic fluorescent tracers are not able to pass (Cook and Oparka 1983; Wang and Fisher 1994; Patrick et al. 1995; Tegeder et al. 1999; van Dongen et al. 2003). The boundary could also be visualized with NMR imaging showing the very different sugar concentration in the endosperm versus the seed coat (Borisjuk et al. 2003).

Interestingly, viruses can enter developing seeds and it was shown in *Pisum sativum* that this happens via a symplasmic pathway (Roberts et al. 2003). In the micropylar region of seeds infected with pea seed-borne mosaic virus, PD-like structures are present between maternal seed coat cells and filial endosperm cells. From the

endosperm, the virus can then traffic symplasmically into the suspensor and on into the embryo. Whether this route is open for nutrients is not clear. No PD have been observed in uninfected cells (Roberts et al. 2003), suggesting that the virus induces the formation of symplasmic connections between seed coat and endosperm.

Sieve element unloading in the single vascular strand that supplies a seed is symplasmic. The sieve elements and companion cells are connected to surrounding parenchyma via PD and tracer diffuses out of the phloem unhindered. In wheat, *Vicia* and pea symplasmic tracers could diffuse all the way to the maternal-filial boundary (Wang and Fisher 1994; Patrick et al. 1995; Tegeder et al. 1999), whereas in *Phaseolus* it only moved into the ground parenchyma (Patrick et al. 1995). Different symplasmic domains in the seed coat are tightly correlated with seed development (van Dongen et al. 2003).

Although seeds from only a small number of plant species were analyzed, it can be assumed that the maternal-filial boundary is generally characterized by the absence of symplasmic connections. Only in case of virus infection, this might change. Furthermore, it is clear that sieve element unloading happens via a symplasmic pathway. The different symplasmic domains that seem to exist both in the maternal and in the filial tissue might play a significant role in seed development.

Fruits develop from different tissues of the flower and have therefore different anatomical preconditions for phloem unloading. Nevertheless, in most cases, a switch to apoplasmic SECCC unloading happens at the beginning of fruit development, like in apple (Zhang et al. 2004), walnut (Wu et al. 2004), and cucumber (Hu et al. 2011), or at a later stage, as in grape (Wang et al. 2003; Vignault et al. 2005; Zhang et al. 2006) and tomato (Ruan and Patrick 1995). In Chinese Jujube varieties, sieve element unloading is apoplasmic at early and late stages but symplasmic at the middle stage (Nie et al. 2010). Strawberry as well is likely to unload apoplasmically at least late in development, indicated by the high apoplasmic sucrose concentration (Pomper and Breen 1995). Similarly, apoplasmic unloading is indicated by sucrose distribution in and around the vasculature in the juicy citrus fruits (Koch and Avigne 1990).

As most fruit accumulate a high amount of soluble sugars, isolation of the SECCC and export of sucrose into the apoplasm can be seen as prerequisite to keep up the sink strength of the organ. Moreover, like it was described for potato development before, the phloem unloading mechanism seems to be an integral part of fruit development, maybe even controlling the progression.

5.3.2 Capacity and Regulation of Phloem Unloading

While regulation of PD function in general is discussed in other parts of this book, its importance becomes especially clear when seen in context of phloem unloading. The distribution of photoassimilates in plants is always tuned to the carbohydrate demand in the different organs. Phloem unloading is the key step in the

control of carbon allocation as it defines the strength of the competing sinks. This is evident in cases where SECCC unloading switches between symplasmic and apoplasmic modes, corresponding to the developmental stage of the sink organ, as seen in stem storage (Viola et al. 2001; Botha et al. 2004), transport stem (Hayes et al. 1987), flower (Werner et al. 2011), and fruit (Ruan and Patrick 1995; Zhang et al. 2006; Nie et al. 2010). At the same time, a change in phloem unloading can be the trigger for a change in sink development (Rinne and van der Schoot 1998; Hancock et al. 2008).

The switch is realized by reduction of PD frequency and/or plugging of the intercellular connections between the SECCC and adjacent cells. Plugging has been assumed in case electron-dense material in PD was observed on transmission electron microscope images, which has been reported in sugarcane storage internodes (Botha et al. 2004), cucumber (Hu et al. 2011), and Chinese Jujube (Nie et al. 2010). The electron-dense material could be protein agglomerations (Ehlers and van Bel 1999).

In grape berries, PD frequency in the SE cell wall remains unchanged during the shift from symplasmic to apoplasmic phloem unloading and only few PD show electron-dense material (Zhang et al. 2006). Here, callose deposition in the neck region of PD causes symplasmic isolation (Vignault et al. 2005). Callose plugs in PD were also identified during development of the Arabidopsis shoot apex (Rinne and van der Schoot 1998).

In addition to blocking, PD were also shown to fine-adjust to carbohydrate demand. In the roots of pea seedlings, treatment with 350 mM mannitol led to an increase of PD neck region and phloem unloading of ^{14}C-labeled sucrose that was applied to the cotyledons (Schulz 1995). Mannitol lowers water potential of the root, thereby increasing sink strength (Schulz 1994). The mechanism behind the dilation of sink PD has been studied in maize and Arabidopsis root apices (Baluska et al. 2001). Based on localization data of the cytoskeletal component actin, the molecular motor protein Myosin VIII, and the Ca-binding calreticulin, which all show specific localization at the outer portions of PD of the unloading zone, the authors propose a hypothetical model for dynamic regulation of PD conductance.

In general, sink PD have a wider SEL than those found in the leaf (Imlau et al. 1999), but the precise capacity of symplasmic unloading pathways has not been conclusively determined, as calculations were based on PD frequencies and presumed effective channel diameters that are unlikely to represent the *in vivo* situation (Bret-Harte and Silk 1994). As an alternative to simple diffusion along the concentration gradient, carbohydrates might move by bulk flow if a strong enough water potential exists between phloem and growth zone. The inclusion of data from measurements of actual *in vivo* cell wall permeability in sink calculations will give a better understanding of symplasmic phloem unloading (Rutschow et al. 2011; Liesche and Schulz 2012b). These experiments will also help to identify compounds and environmental factors that influence PD conductivity and thereby the capacity of the unloading pathway as it was shown for H_2O_2 (Rutschow et al. 2011).

5.4 Perspectives on Phloem Loading and Unloading

Phloem loading and unloading are essential processes in plant carbon allocation and therefore prominent targets for crop plant enhancement (Ainsworth and Bush 2011). Understanding the mechanisms and pathways of whole-plant carbon transport will also help us to estimate the potential effect of climate change (Litton et al. 2007).

The recent progress in phloem loading research led to appreciation of mechanistic diversity. The complex picture we have now enabled us to reconsider the purpose of phloem loading (Turgeon 2010), linking it to plant growth forms and ecological parameters (Davidson et al. 2011). At the same time there is a better understanding of how Münch's pressure flow mechanism is implemented in different plants with a wide anatomical variety (Liesche and Schulz 2012a).

Nevertheless, important questions remain. Especially important is the question of how phloem loading is regulated. How are signals from the sink, whatever they are, received and relayed in source? The symplasmic connection between the phloem SECCC and surrounding leaf cells might play a crucial part, potentially enabling passage of small signaling molecules or even electric signals transmitted in the plasma membrane (Furch et al. 2007). The signaling function of PD at this interface might be the reason that they are present even in plants that use the active apoplasmic loading phloem mechanism, where symplasmic coupling is counterproductive and potentially harmful for plants as they enable viruses to spread systemically.

Also related to regulation is the question of how the phloem loading rate is adapted to the environmental and developmental conditions. At least in the apoplasmic loaders are sucrose transporters and sucrose efflux carriers likely candidates. Sucrose transporters were shown to be subject to regulation at transcriptional, posttranscriptional, and posttranslational level (Liesche et al. 2011a). Sucrose transporters could also play an important role in regulation of phloem loading in the symplasmic loaders, as the vacuolar sucrose transporters clearly have an effect on carbohydrate export (Eom et al. 2011; Payyavula et al. 2011). So far, only sucrose symporters have been identified, which could facilitate sucrose transport out of the vacuole, but it has been suggested that tonoplast-localized glucose antiporters that also transport sucrose could be responsible for import into the vacuole (Schulz et al. 2011).

Plants using active symplasmic phloem loading were shown to not be able to increase carbon export under improved light conditions as active apoplastic loaders do, which was explained with their inflexible anatomy, i.e., the fixed number of PD available for transport into the SECCC (Amiard et al. 2005). Comprehensive modeling of sugar flux through the specialized PD at the BSC–IC interface and *in vivo* studies with tracers of different size would help to elucidate the specifics of this polymer trap mechanism and the limitation and benefits it offers for the plants that employ it.

The mechanism of plasmodesmata function and their role in the regulation of phloem loading is poorly investigated. On one hand, it has been shown that pressure

difference and other factors can lead to increased plasmodesmal resistance, blocking the transport of tracer molecules (Oparka and Prior 1992). On the other hand, plasmodesmal conductance can also be increased in response to osmotic stress as shown for PD in the post-phloem pathway of pea seedling roots (Schulz 1995). Dynamic regulation of plasmodesmal conductance would have important implications for all phloem loading strategies as it would directly affect the capacity of symplasmic pathways.

Another fundamental question that has hardly been addressed is how the outward water movement from xylem towards the stomata affects the inward sucrose diffusion. Especially in symplasmic loaders, without a sucrose pumping mechanism, water movement could slow down diffusion if it is not confined to separate pathways. But water could also help to move sucrose towards the phloem by preferentially entering the mesophyll cells, the putative site of highest sugar concentration in symplasmic loaders. Water uptake would lead to increased osmotic pressure that could create bulk flow through plasmodesmata towards the phloem. Measurement of intracellular sucrose concentration and turgor pressure, as well as visualization of water movement, for example, by quantification of aquaporins in the plasma membranes of the different cells, is needed to understand the interaction of water and sugar pathways in leaves.

Gymnosperms were recently shown to utilize the same basic mechanism for phloem transport as angiosperms (Jensen et al. 2012). Also regarding phloem loading, there are many similarities to the passive symplasmic loading angiosperms, suggesting an implementation optimized for trees. It remains to be seen if gymnosperm phloem loading is indeed similar to the passive symplasmic phloem loading of angiosperm trees or if their unique anatomical features, like endodermis with Casparian strip and the transfusion tissue, put them in a group on their own.

As gymnosperms developed mostly in parallel to angiosperms, it cannot be concluded from their symplasmic loading that this is the most ancient loading strategy. Extensive research into phloem loading in pteridophytes will be necessary to come closer to a solution to that question. The rapidly increasing availability of genomic data will enable conclusive phylogenetic analysis of sucrose-transporter proteins that might provide important insight already in the near future.

Our understanding of phloem unloading is still somewhat limited by the small number of plant species in which it has been studied. In some sink tissues, like bean seed or Arabidopsis root, the mechanism has been comprehensively elucidated, but generalization of the results is dangerous because of the anatomical and developmental diversity of the sink organs between different plant species. Seed loading, for example, always involves one apoplasmic step, as no PD exist between maternal and filial tissue, but the unloading pathway up to the seed coat can be symplasmic or involve apoplasmic steps at the SECCC–BSC interface or other interfaces further down the pathway, depending on plant species (Patrick and Offler 2001; Werner et al. 2011). The variety in unloading strategies is even wider in fruits, which is not unexpected considering the big differences in fruit anatomy. Therefore, the exact specifications of phloem unloading have to be carefully investigated in each tissue.

The formation of symplasmic domains are emerging as integral part of organ development. Whereas complete symplasmic phloem unloading was long assumed to be the major route in all sinks except embryonic tissue (Patrick 1997), more recently, distinct symplasmic domains have been discovered in various tissues and their occurrence was shown to coincide with important transitions in organ development (Zhang et al. 2006; Hancock et al. 2008; Werner et al. 2011).

The quantification of phloem unloading capacity will be important for whole-plant modeling of carbon allocation patterns, since under most environmental condition, the sink strength determines carbon distribution (Minchin and Lacointe 2005). Live-cell imaging has become an important tool for the quantification of intercellular movement of molecules, and many more results regarding flux capacity and regulation can be expected (Rutschow et al. 2011; Liesche and Schulz 2012b).

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Chapter 6
Mechanism of Long-Distance Solute Transport in Phloem Elements

Craig A. Atkins

Abstract Descriptions for the C/N economy and translocation of solutes in species of lupin (*Lupinus albus* [L.] and *L. angustifolius* [L.]) are used to exemplify the "source/sink" relationships of a grain legume. Identification of translocated solutes requires invasive sampling from sieve elements of phloem, and techniques for collection of exudates and limitations in interpreting data are discussed. Mechanisms that fashion the solute composition of phloem are described using the development of pods and seeds as a specific example. In lupin, the two predominant solutes of phloem are sucrose and asparagine and molecular explanations for selectivity in the translocation of these commodities are identified. Critical components such as membrane transporters for both loading and unloading sucrose and asparagine from "source" organs and into "sink" organs, respectively, are described together with the nature and regulation of metabolic pathways for their utilization.

Keywords Lupin • *Lupinus* • Source/sink relationships • Phloem • Translocation • Transport

Abbreviations

DAA	Days after anthesis
asn	Asparagine
gln	Glutamine
asp	Aspartic acid

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glu	Glutamic acid
SE	Sieve element
CC	Companion cell
ASNase	Asparaginase

6.1 Introduction

The two long-distance transport pathways in flowering plants are structurally co-located in the vascular network and in many cases are separated by just a few files of parenchyma or other non-transport cells. However, they are quite dissimilar in how each contributes to the nutrition of the plant and in the processes that load and unload solutes to and from the translocation streams they support. The transpiration stream that transfers water and solutes from the soil as well as products of root (and nodule in the case of legumes) metabolism to shoot organs occurs in the dead cells of xylem and is essentially acropetal. Evaporation of water at the transpiring surfaces of the plant creates a negative tension that permits deposition of substances dissolved in the contents of xylem. Typically xylem contents are a dilute solution with an acidic pH. On the other hand phloem constitutes living cells that provide a symplasmic route for translocation of solutes from sites of synthesis ("sources," mainly leaves) to sites of their utilization in growth and development ("sinks," such as roots, meristems, and reproductive organs). Translocation in phloem may be acropetal or basipetal. The solutes in phloem are much more concentrated, are dominated by sugar which in many cases is sucrose, and are neutral to alkaline in pH. However, each of these streams also contains a myriad of both low molecular weight solutes as well as macromolecules, many of which are present in trace quantities and which may serve roles other than nutrition (Atkins and Smith 2007; Atkins et al. 2011). Knowledge of mechanisms that underlie how the "source/sink" relationships of a plant are established requires sampling translocation streams to document the solutes that provide nutrition during development. Inventories of translocated solutes have been generated from a number of species but among legumes lupin species (*Lupinus albus* and *L. angustifolius*) provide a unique resource in this respect, and data from these crop species is used to describe how source/sink relationships are formed and regulated at the molecular level.

6.2 Compiling Inventories of Translocated Solutes

Inventories of translocated solutes, whether in the transpiration stream or in phloem, have been compiled from analyses of exudates or "saps" collected following vascular tissue disruption. The least invasive is the use of severed stylets of sap sucking insects that specifically invade sieve elements (SE) of phloem, although recent

evidence indicates that under some circumstances aphids also feed from xylem to obtain water and maintain their osmotic balance (Pompon et al. 2011). However, yields of exudate from stylectomy are very low and a limited number of plant species have proved amenable. Thus, most data comes from exudates collected following incision of the vasculature, i.e., from a wound. While substantial volumes of exudate have been collected in this way, damage to cells other than SE unavoidably leads to contamination. These aspects are considered in detail elsewhere (Atkins et al. 2011) but suffice to say that for the major low molecular weight solutes, particularly those of C and N, contamination is likely to be slight, while for solutes in trace concentrations a high degree of caution is needed in assigning their presence in exudates to the contents of SE and to translocation. There is a growing body of evidence for specific "signaling" functions of the translocation streams and especially for phloem. Putative signals range from low molecular weight solutes like sugars and particular amino acids to peptides, proteins, and a range of RNA species including mRNA and miRNAs (Atkins and Smith 2007; Rodriguez-Medina et al. 2011; Atkins et al. 2011). The sites of synthesis and function of these many and varied macromolecules have yet to be discovered and, except for a few, their translocation remains to be demonstrated.

Plants that "bleed" spontaneously from incisions to their vasculature have proven to be particularly valuable in building up a picture of the solutes likely to be translocated. These comprise a diverse group of species that includes castor bean (*Ricinus communis*), a number of cucurbits (pumpkin, squash, and cucumber), the developing floral apex of rapeseed (*Brassica napus*), and a number of members of the genus *Lupinus*. Interestingly, among the lupins only some species are known to "bleed" their phloem. These are *L. albus*, *L. angustifolius*, *L. mutabilis,* and *L. cosentini*. While there has been considerable progress in describing processes that occlude sieve plates and restrict loss of SE cell sap, the molecular mechanisms involved remain subjects of speculation (Froelich et al. 2011; Knoblauch and Oparka 2012; Walker and Medina-Ortega 2012), and reasons why some species "bleed" freely is not clear. In a number of legumes, phloem occlusion is believed to involve Ca^{2+}-dependant subcellular contractile bodies, termed "forisomes," that form plugs and potentially block SE on wounding (Knoblauch et al. 2012). Forisomes are also present in images of SE in *L. albus* (Rodriguez-Medina 2009) but whether their function is impaired following wounding remains unknown.

A considerable body of data on phloem mobile solutes as well as studies of the mechanisms involved in the translocation process has come from the use of severed cucurbit stems. However, recent studies (Zhang et al. 2012; Zimmermann et al. 2013) of the origin and composition of exudate in pumpkin and cucumber together with a detailed morphometric analysis concluded that while the exudate contained solutes from SE, it was diluted by water from surrounding cut cells, by the apoplasm, and by exudation from severed xylem. Consequently, the concentrations of sugars (stachyose, with lesser amounts of sucrose and raffinose) in cucurbit exudates are typically 1–5 mM (Zhang et al. 2012). By comparison, concentrations of sucrose in exudate from *Lupinus albus* varies from 0.3 to 0.6 M depending on the

site of collection (Pate et al. 1979); values approaching those measured by aphid stylectomy in other species (Riens et al. 1991; Voitsekhovskaja et al. 2006) and likely to more closely reflect the concentration being translocated.

Because of a wealth of genomic data available for the model species, *Arabidopsis thaliana*, much of our knowledge about the likely transporters involved in translocation have come from studies of the species (Tegeder et al. 2011). However, *Arabidopsis* does not freely exude from a wound and collection of solutes involves bathing the wound in a buffered solution containing EDTA (Lam et al. 1995; Zhang et al. 2010; Guelette et al. 2012; Gaufichon et al. 2013). Consequently, the collection liquid provides relative concentrations of solutes and the likely consequences for contamination using this technique in a number of species have been discussed elsewhere (van Bel and Hess 2008; Atkins et al. 2011; Liu et al. 2012; Guelette et al. 2012). Perhaps the most definitive N solute analysis in phloem of *Arabidopsis* comes from exudate collected by aphid (*Myzus persicae*) stylectomy (Hunt et al. 2010) showing that the dicarboxylates aspartate (asp) and glutamate (glu) were dominant N solutes rather than their amides asparagine (asn) and glutamine (gln).

Harris et al. (2012) have recently compiled a large data set for the amino acid composition of phloem from 28 published reports that included 52 analyses of which 25 were of undiluted exudate (from stylectomy and freely exuding wounds) and 27 of diluted solutes collected into EDTA solutions. The relative composition of amino acids was comparable for the two types of collection except for asn, which was much higher in the diluted exudate (16.67 ± 19.23 %) compared to the spontaneous exudate (6.07 ± 6.35 %). From these data Harris et al. (2012) calculated the average concentration of the 20 protein amino acids in phloem and found glu and gln each around 28 mM and asp and asn each around 16 mM as the major contributors to the total amino acid content which, on average, was 150 mM. Interestingly, the information used in this compilation was predominantly from monocotyledon species and included limited data from legume species and none from *Lupinus*.

6.3 Translocation of C and N in *Lupinus albus*

While the following considerations of mechanisms that fashion the contents of phloem streams are idiosyncratic for lupin species, the broad aspects of the partitioning of C and N that are predicted from an accounting of translocated solutes to satisfy the growth of component organs of the plant are likely to have wider application. Indeed Peuke (2010) has recently reviewed and reevaluated the use of similar experimental methods and models of nutrient flow to determine source/sink relationships in castor bean.

Experimentally derived data on C, N, and H_2O balances for the organs of white lupin throughout development together with changes in the C/N weight ratios of solutes in xylem sap collected from roots and stem segments and in phloem exudates collected from a number of sites on the shoot (stem base, mid stem and upper stem, petioles, the stylar tip, and suture vasculature of developing fruits)

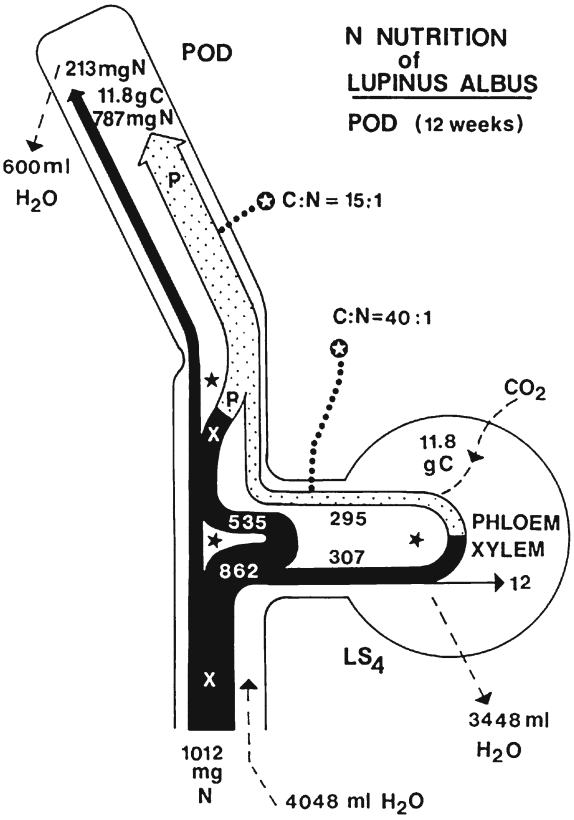

Fig. 6.1 Schematic depiction of the exchanges of solutes (as amounts of water, C, and N) between xylem and phloem translocation streams in the uppermost leaves and stems of a fruiting main stem shoot of *Lupinus albus*. The shoot comprised four mature leaves in the upper stratum (LS4) and three developing fruits. The period of development (POD) spanned 12 weeks from antithesis to physiological maturity just prior to pod desiccation [Data derived from Layzell et al. (1981) and modified from Atkins (1993)]

have been used to construct empirical models of solute flow (Pate et al. 1979; Layzell et al. 1981). The assumptions and calculations underlying the models are outlined in Pate et al. (1980). These models have identified a number of specific solute exchanges that together account for the phloem contents that satisfy the nutritional demands of individual developing organs for C and N but which also account for the distribution of inorganic nutrients (Jeschke et al. 1985), plant growth regulators (Emery et al. 2000), and translocated quinolizidine alkaloids (Lee et al. 2007). While flows of solutes have been generated for all the organs of the growing white lupin plant, a useful example that indicates the relevance of solute exchanges/transfers is the developing fruit and its supporting vegetative structures of the upper stem (Fig. 6.1).

The xylem supplies the shoot at the site of the uppermost mature leaf stratum with 4 L H_2O and a little more than 1 g N (Fig. 6.1) during 12 weeks of development. The majority of this N is in the form of asn with a lesser quantity of gln and smaller amounts of other amino acids (Atkins et al. 1975), and, even though 307 mg N is directed to the leaves, almost all is translocated away in phloem together with 11.8 g C, largely as sucrose, assimilated by the leaves through photosynthesis.

Phloem exudate collected at the petioles of these leaves has a C/N ratio of 40:1, but by the time the translocation stream has traversed, the upper stem segment and the rachis of the inflorescence addition of N lowers this to a C/N ratio of 15:1, a composition that more closely matches the measured C and N nutrition of the fruit. Fig. 6.1 describes the addition of N to the phloem as transfer from xylem in the stem. Thus, three exchanges between vascular components are envisaged as essential mechanisms in the overall C and N nutrition of the fruit. The first is enrichment of xylem with N as it traverses the stem through a greater than proportional flow of water to the major transpiring surface of the leaves. In fact analysis of xylem contents displaced by gentle vacuum from segments of main stem towards the apex shows that the N concentration increases progressively and is fivefold at the apical stratum of leaves compared with incoming sap from the nodulated root system (Layzell et al. 1981). This interfascicular transfer of xylem-borne N in the stems is envisaged, rather hypothetically (as in Fig. 6.1), as feeding back into the upward transpiration stream. The second is the almost direct transfer of N from the xylem in the leaf to the exiting phloem stream and the third is the further enrichment of phloem with xylem-borne N also in the stem as noted above. These solute transfers between xylem and phloem are quantitatively the major mechanisms that fashion the composition of phloem providing an appropriate C/N ratio to the nutrition of the developing fruit and, as indicated in the following discussion, are dominated by asn.

Whether the exchanges of solutes are symplasmic, apoplasmic, or a combination of both pathways, bidirectional radial solute movement between xylem and phloem streams in stems has been established as an important component in the distribution of nutrients within the plant (Biddulph and Markle 1944; van Bel 1990). The degree to which N solutes in transit mix with the soluble pools of amino compounds outside the vasculature in the stem is not known. However, these pools and particularly those of the dominant N solutes are readily available to the phloem in the stem.

In nodulated lupin the translocation of N throughout the plant is dominated by the two amides, gln and asn (Pate et al. 1979). Asn in xylem leaving the nodules and the root system carries over 90 % of the translocated N and, although this diminishes to around 60–70 % in phloem exudate collected from stems, petioles, and developing fruits, the two amides together account for 80 % or more of phloem-borne N throughout the life cycle of the plant. The predominance of asn is not restricted to symbiotically dependant plants. Non-nodulated lupin supplied NO_3 in the rooting medium translocated 50–70 % N as asn in xylem and 70–80 % N in phloem (Atkins et al. 1979). Except for species in the *Phaseoleae* that fix atmospheric N_2 in nodules to form ureides, the overwhelming majority of legumes form and translocate asn (Atkins 1991). In non-leguminous plants the amides are also prominent forms of translocated N but as noted above in *Arabidopsis,* the dicarboxylates may dominate in some species.

Xylem/phloem transfer in mature white lupin leaflets has been studied using ^{15}N- and ^{14}C-labeled amino acids supplied in the transpiration stream of detached fruiting shoots (McNeil et al. 1979; Atkins et al. 1980). Asp and glu entered leaf mesophyll and metabolic pathways that used their C and N to form other compounds including a range of amino acids, some of which were subsequently loaded

onto the phloem and exited the leaf. On the other hand gln and asn delivered to the leaf in xylem were not extensively metabolized and were recovered in phloem exudates essentially unmetabolized. In the case of asn, the same $^{15}N/^{14}C$ ratio was measured in petiole and fruit exudate as that in labeled asn supplied in the transpiration stream (Atkins et al. 1980) providing direct evidence that the major incoming N solute, asn, did not equilibrate with unlabeled pools in the leaf and was excluded from the amino acid metabolism of the mesophyll. Autoradiographs of leaflets following the short pulse of ^{14}C asp and asn followed by a 2-h "chase" with unlabeled asp or asn showed only that the vein network of the leaf was labeled in the case of asn but was cleared of label in the case of asp which showed that the ^{14}C had accumulated outside the veins in the leaf mesophyll (McNeil et al. 1979).

While this picture above applies to the mature leaf, C and N nutrition varies throughout the period of development. Detailed study of the C and N economy of a leaf of white lupin from 1 to 66 days after emergence identified four distinct ontogenetic phases (Pate and Atkins 1983). From 1 to 11 days, the leaf imported both C and N in phloem and xylem, and from 11 to 20 days, this became massive for xylem-borne N as the leaf expanded, transpiration increased, and the leaf became a net exporter of C as a consequence of photosynthetic C assimilation. At this stage, of the 5.8 mg N imported in the transpiration stream, close to 20 % was transferred to phloem and exported, the remainder being used for leaf growth together with 28 % of the net C gain. From 20 to 38 days, some N mobilization from the leaf was recorded, and while 3.8 mg N entered, 4.6 mg N exited in phloem together with 99 % of the net C gained in photosynthesis. Finally, as the leaf senesced (38–66 days) and photosynthetic C gain decreased, 6.5 mg N entered in xylem and together with nearly 2.5 mg N mobilized from the leaf exited in phloem. The solutes involved in these N flows were documented by analysis of xylem (upper stem tracheal sap) and phloem exudates (from petioles) and of changes to the soluble and protein amino acid pools in extracts of the leaf tissue throughout development (Atkins et al. 1983). Small transient changes in the amino acid composition of both translocation streams were recorded but asn and gln were the major N solutes throughout, and during the first 1–11 days, they were delivered in excess of their utilization in soluble and protein contents of the leaf. Fifteen other amino acids in xylem and phloem were delivered in amounts that required metabolism of the amides to satisfy protein synthesis. During this period asparaginase (ASNase) activity was maintained at a high level. Asn-dependant synthesis of amino acids continued in the period from 11 to 20 days but with an increasing amount of asn and gln exported in phloem. By 20 days when asn metabolism to provide protein amino acids to the growing leaf declined, the rate of ASNase activity declined by 80 % and higher levels of asparagine aminotransferase (AAT) activity were recorded. Not surprisingly the leaf showed significant capacity for ammonia assimilation by glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) over the whole 66 days of development. During the period of maximum rates of photosynthesis (20–38 days), xylem inputs of asn and gln accounted completely for the outputs of these amino acids in phloem, while in the period from 38 to 66 days, there was significant mobilization and export of N as asn but more significantly as gln in phloem.

The exchanges of N in the stem that enrich the N content of phloem destined for the fruit are also closely linked to phloem loading of asn. The specific activity of uniformly ^{14}C-labeled asn supplied in xylem to cut fruiting main stem shoots of white lupin (as described in Atkins et al. 1980) was unchanged in phloem exudate collected at the petioles of mature leaves but was diluted significantly by "cold" asn when analyzed in exudate collected at the fruit. While the source of this asn loaded onto phloem in transit could have been in the stem segments or the inflorescence stalk or associated with the fruit vasculature, it indicates that the available asn pool is not simply stored in these tissues but does contribute to the translocation stream moving towards a strong sink. Autoradiographs of stem and petiole material made in these labeling experiments indicate that for asn, gln, and valine but not for some other amino acids (asp, glu), the nodal tissue vasculature was heavily labeled (McNeil et al. 1979). These are sites where extensive xylem parenchyma transfer cells have been documented (Gunning et al. 1970), and while direct evidence for their role in retrieving xylem N remains to be established, it is tempting to assign such a role. However, the identification of similar cell profiles in the internodes of some 21 legume species, including white lupin, suggests that xylem enrichment with N is likely throughout the stem (Kuo et al. 1980). Non-nodulated *Phaseolus vulgaris* seedlings deprived of combined N around their roots or supplied with N as a foliar spray failed to differentiate these internode transfer cells indicating that their formation is closely linked to N translocation (Kuo et al. 1980). Whether there are specific effects of particular N solutes in inducing their development, for example, asn have not been explored. Major transient changes to the N nutrition of plants has been shown to result in extensive adjustment of gene expression (Scheible et al. 2004), and it is not surprising that this extends to the differentiation of specialized cells which might satisfy the need to enhance transport processes.

The foregoing shows that asn synthesis, its transport, and metabolism are an important and central feature of N nutrition in lupin. While the molecular and metabolic mechanism(s) which fashion the N composition of phloem leaving a lupin leaf have not been resolved, differences in the pathway of phloem loading for different solutes can be inferred from data for other species. Gessler et al. (2003) also used double-labeled amino acids in studies of long-distance transport of N in spruce trees and found evidence for the exchange of these compounds between xylem and phloem. They further found that the two amino acids, gln and asp, behaved quite differently, possibly due to differences in loading onto the translocation streams. Komor et al. (1996) have emphasized the fact that pathways involving mesophyll cell connections are likely to be different in different plant species. Such specific interchanges are not restricted to amino acids. Schneider et al. (1994) found evidence for reciprocal radial exchange of the peptide glutathione between the translocation streams in spruce seedlings as a central feature of S nutrition in the species. Similar conclusions have been made for the recirculation of K^+ and Mg^{2+} in nodulated white lupin (Jeschke et al. 1985) and in the translocation of Zn^{2+} in the peduncle of wheat (Herren and Feller 1994). Such transfers between the two long-distance transport pathways are clearly significant in the nutrition of plant organs and particularly in fruit and grain development.

6.4 Molecular Mechanisms in the Translocation of N Solutes

As noted above the fashioning of phloem N composition in lupin is dominated by transfers of the amides gln and asn, and especially asn. Recent studies in *Arabidopsis* suggest that asn synthesis is not only central to N metabolism but is essential for effective N translocation even though this amide is not the dominant phloem mobile N solute in the species (Hunt et al. 2010). Gaufichon et al. (2013) generated knockout lines for the most highly expressed asparagine synthetase (ASN2) gene and showed that the mutant plants had a defective growth phenotype with severely reduced levels of the amide in leaves and also in phloem exuded into an EDTA solution. *ASN2* expression was localized specifically to phloem CC and the authors concluded that ASN2 was "essential" for phloem loading and maximizing translocation of N. Thus, in *Arabidopsis* as in lupin, asn appears to serve a central regulatory role in N translocation. While these data are not definitive proof that asn fulfills this role, in the knockout line there was no compensatory increase in gln in phloem, despite an increased level of ammonia in the leaves, and the plants suffered N deficiency. Interestingly, $^{15}NO_3$ uptake by roots was reduced in the mutant indicating that impaired asn synthesis had a profound negative effect on the overall N economy of the plant. If indeed maximizing translocation of N requires asn synthesis, then molecular mechanisms responsible for or which regulate its transport at the cellular level in the translocation pathways are likely genetic targets to enhance the efficiency of nutrient use in enhancing yield potential.

Although asn synthetase knockouts have not been generated in lupin, a study in which asn synthesis in nodules was eliminated by transient exposure to a rooting atmosphere devoid of N_2 (80 % Ar: 20 % O_2) showed that N assimilation could be altered, at least transiently, in favor of gln synthesis and translocation (Atkins et al. 1984). Following 3 days in Ar/O_2, asn synthetase activity in nodules could not be detected and, even after returning these plants to a rooting atmosphere of air, activity only increased to a fraction of controls maintained in air throughout. On the other hand GS activity was unaffected and measurable GOGAT activity was retained. Thus, at least for short periods gln could replace asn in the N economy of the plant.

A recent review (Tegeder 2012) has highlighted the current lack of knowledge about regulation of amino acid transport and of the transporters likely to be involved in the many transfers of C and N described above for lupin. A wide range of sugar transporter proteins have been described from a number of plant species, including *Arabidopsis,* with the majority functioning as plasma membrane-localized H^+ symporters (reviewed in Tegeder et al. 2011). Among these a group of sucrose transporters (SUTs or SUCs) are associated with phloem and in some cases specifically with CC or SE. Their likely function in phloem loading/unloading of sucrose has been confirmed in studies using mutation strategies and RNA interference, and in a number of cases, alterations in expression have led to significant changes in the development of both vegetative and reproductive sink organs. Interestingly, AtSUC2 is expressed in the transport phloem and is thought to be

important for the efflux/retrieval of sucrose (Gould et al. 2012) and in this respect provides a potential means to alter solute composition of phloem in its passage towards sink organs.

The majority of data relating to the identification of proteins mediating the movement of organic N compounds has come from studies with *Arabidopsis* (reviewed in Rentsch et al. 2007; Tegeder et al. 2011; Tegeder 2012) with some 60 putative amino acid transporters (ATFs) identified in the species to date. Among these, the amino acid permeases (AAP1-8) mediate H^+-coupled transport of acidic and neutral (including asn and gln) amino acids and have been localized to a number of cell membranes. In *Arabidopsis* AAP2 has been localized to CC of phloem throughout the plant and *AAP2* T-DNA insertion lines show changes in the translocation of amino acids consistent with this transporter functioning in phloem loading (Zhang et al. 2010). In *aap2* mutants total amino acids were significantly reduced in phloem (based on exudation into an EDTA solution) due largely to a decrease in gln, while levels of other amino acids were unaffected. The *aap2* mutant lines showed changes in leaf C/N balance with greater leaf area, increased photosynthetic rates and enhanced expression of enzymes associated with sugar metabolism. Changes in leaf metabolism resulted in increased branch and silique number and as a consequence enhanced grain yield. A central role for xylem/phloem solute exchanges mediated through AAP2 was inferred and, while the transporter undoubtedly is important in determining the N economy of the plant, a specific role with respect to a particular amino acid was not established. While AAP2 has a modest affinity for a range of amino acids, including gln, a relatively high concentration of the amide ensures high throughput, and this may also be the case for the many legume species which, like lupin, have asn as the predominant N solute in phloem (Atkins 1991). An insertional inactivation mutant for the AAP6 amino acid transporter gene in *Arabidopsis* resulted in phloem exudate amino acid levels reducing more than threefold compared to exudate from wild-type plants (Hunt et al. 2010). Importantly SE exudate was collected by aphid stylectomy and in samples from wild type, the dicarboxylates asp and glu were the predominant N solutes. Although considerable variance in the levels of 16 amino acids resulted in only some being significantly different, in fact the levels of all were reduced with asp reduced by more than 80 % in stylet exudate from the mutant, and this is consistent with AAP6 having a higher affinity for asp than other AAPs (Fischer et al. 2002). Interestingly, the phenotype of the *aap6* mutant plants showed no obvious developmental differences to the wild type except that the seeds were slightly larger in the mutant. Expressed in *Xenopus* oocytes, AAP6 shows a higher apparent affinity for asn ($K_{0.5} = 0.36$ mM) than asp ($K_{0.5} = 1.64$ mM) and a value about one tenth that determined for AAP1-5 (Table 2 in Fischer et al. 2002), suggesting that perhaps this AAP is specifically associated with the transport of asn in lupins. However, other transporter molecules with high affinity for a range of amino acids have also been localized to the plasma membrane in *Arabidopsis* (Lee and Tegeder 2004) so that there may be a range of molecular mechanisms regulating transport of N solutes in the long-distance translocation pathways.

6.5 Translocation to and Within Developing Seeds

There has been considerable attention paid to the features of amino acid transport to and within legume seeds where high rates of protein synthesis are supported by translocation of N solutes largely through phloem. Both the amide-N and C of incoming double-labeled asn were widely distributed in the soluble amino acid pools and in most amino acids of the storage proteins of the lupin embryo during the cotyledon filling stage (Atkins et al. 1975), and, given the high concentration of asn translocated to the developing fruit, these outcomes are what might be expected.

In *Arabidopsis,* AAP1 and AAP8 have been associated with transport activity in seeds (Schmidt et al. 2007; Sanders et al. 2009) and in *ataap8* mutants a significant rate of embryo abortion occurs but amino acid and protein reserve levels are unaffected. AAP1 expression has been localized to the developing embryo in *Arabidopsis* seeds, and *ataap1* mutants show considerable changes in N distribution with greatly increased accumulation of amino acids in the seed coat and endosperm (Sanders et al. 2009) consistent with decreased uptake from the apoplast. The most significant accumulation was gln which increased more than 20-fold compared to wild type. The mutation was accompanied by significant changes in expression of genes involved in N metabolism. Many were reduced (GS, GOGAT and ASNase) but asn synthetase transcript was increased by as much as threefold. Seeds were smaller and overall yield was reduced indicating a close relationship between the effective transport of N solutes and storage protein reserve synthesis. It has been argued that similarity in amino acid composition in xylem, phloem, and surrounding mesophyll cytosol indicates that amino acid transport into and from the transport channels is nonspecific. However, individual AAPs isolated from *Arabidopsis* exhibit very diverse affinities for specific amino acids (Fischer et al. 2002), and even though there is limited data for mutants with altered expression of some of the transporters, it is clear that there are consequences for N translocation when their expression is impaired.

Only *AAP1* and *AAP2* have been cloned and sequenced from lupin (*L. angustifolius*; Ferguson 2005). Each showed a high degree of sequence identity with other AAP transporters, and hydropathy profile analysis indicated both encode highly hydrophobic proteins likely to have 11 transmembrane domains with a cytosolic N-terminus. Real-time RT-PCR analysis showed that while each was expressed in all vegetative and reproductive tissues, *LaAAP1* transcripts predominated in flowers and young expanding whole seeds, while both were expressed at similar levels in older seeds when cotyledons were filling rapidly. Among reproductive tissues the highest levels of expression for both transcripts was in pod walls and, although each was found in both seed coats and developing embryos, *LaAAP1* was expressed at higher levels and increased sharply in embryos during seed filling (Ferguson 2005). Whether or not these transporters regulate rates of transport of amino acids in lupin seeds is not known but AAP1 has a very low affinity for asn (Fischer et al. 2002), and in a yeast complementation study (Ferguson 2005), neither LaAAP1 nor LaAAP2 transported glu but LaAAP1 could transport gln measured as ^{14}C [U] gln uptake.

In experiments similar to those described earlier, ^{15}N [amide N]$^{-}$/^{14}C [U]-labeled asn was supplied to cut fruiting shoots of white lupin in the transpiration stream and the identity of labeled metabolites that accumulated in the seeds of a developing fruit determined (Atkins et al. 1975). The amide N was released through activity of an asparaginase (ASNase) and, for some 4 weeks of development, ammonia (NH_4^+) accumulated in the liquid endosperm (up to 6 μl seed^{-1}) reaching levels as high as 70 mM and heavily labeled with ^{15}N. Similar high concentrations of NH_4^+ have been recorded for the endospermic fluid collected from pea (*Pisum sativum*; Murray 1980), in which the volume of endosperm reaches as much as 35 μl (Marinos 1970), and common bean (*Phaseolus vulgaris*; Smith 1973). The most abundant amino acid in lupin endosperm was alanine (and also in bean, Smith 1973) which was a minor constituent of incoming phloem indicating major metabolism of N in the seed coat. These data raise interesting questions about the role of NH_4^+ accumulation in endosperm and particularly about transport processes both in unloading asn into the seed coat and its metabolites from the seed coat into the apoplast and the uptake of N solutes, including NH_4^+, by embryo tissues.

In addition to serving as a source of N, there is some evidence that NH_4^+ has a specific role in embryogenesis (Halperin and Wetherell 1965) but supporting *in planta* data have yet to be documented. The amino acid composition of endospermic fluid is thus quite different to that of incoming phloem (Atkins et al. 1975; Murray 1980) and this is also true for the mineral composition (Hocking and Pate 1977) and the sugars that accumulate (Smith 1973; Murray 1980). As a consequence of the expression of a cell wall-bound invertase in the seed coat, hexoses (glucose and fructose), rather than sucrose, are unloaded into and accumulate to high levels in the endosperm (Patrick and Offler 1995). In bean the levels of reducing sugar in this compartment reach more than 60 mM (Smith 1973) and in pea endosperm levels of 76 mM have been recorded (Murray 1980). Sucrose hydrolysis through invertase occurs at a time when embryo development is characterized by cell division and it is believed that sugars in this form promote and prolong this phase of embryo growth (reviewed in Weber et al. 1997). However, later during seed filling sucrose rather than the hexoses are transported to the embryo, and metabolism through sucrose synthase (SuSy) and sucrose-phosphate synthase, rather than through invertase, predominates.

In situ hybridization studies of ASNase-GUS fusions in developing *L. angustifolius* seeds (Grant and Bevan 1994) indicated expression in the seed coat of relatively young [4–17 days after anthesis (DAA)] seeds. Not the seed coat endothelium, the endosperm or the embryo showed transient expression. However, a more detailed study of ASNase expression during seed development from 25 to 51 DAA in the species found that while a high level of transcript was present in the seed coat, expression could also be detected in the embryo but not the endosperm (Ferguson 2005). This period included the phase of development when a liquid endosperm was present (7–35 DAA) and suggests that significant asn hydrolysis occurred in the seed coat with the resultant NH_4^+ formed transferred to the endosperm where there was negligible ASNase transcript or activity. A putative NH_4^+ transport protein gene (*LaAMT1*) with sequence homology to a number of *AMT1* genes from different

plant species and cloned from *L. angustifolius* vegetative tissues was used to probe seed tissues. A high level of the transcript was detected in the seed coat but was negligible or not detectable in the embryo or the endosperm (Ferguson 2005) throughout the 25–51 DAA period. High levels of GS expression were found in both seed coat and embryo but not in the endosperm, and one of the storage proteins, conglutin γ (Foley et al. 2011), was expressed exclusively in the embryo throughout this period, consistent with the onset and progression of seed filling. Thus, it seems likely that a significant portion of incoming translocated asn is hydrolyzed to release NH_4^+ which is transported from the seed coat to endosperm. Because of the dominance of asn in the nutrition of the developing seed and the metabolic pathway in seeds that relies on ASNase, attempts were made to genetically engineer lupins to modulate expression of the enzyme (Ferguson 2005).

L. angustifolius transformants containing a sense copy of the lupin ASNase gene driven by the lupin conglutin γ promoter showed significantly higher ASNase expression in both seed coats and especially in embryos compared to wild-type or a negative control transformed with a vector lacking the gene construct. Four of 16 transgenic lines also showed small but significant increases in mean seed weight and seed yield. An antisense construct for ASNase (sense-intron-antisense hairpin), also driven by the conglutin γ promoter, was used to transform plants to reduce expression of the enzyme. The transcript both in seed coat and embryo tissue was reduced to extremely low levels, and pod set on the primary inflorescence was markedly reduced. However, pod set was greater on the inflorescence branches so that overall there was no significant difference in mean seed weight or total seed yield compared to wild type or to a negative control. These data suggest that either asn deamidation is not critical for seed development or an alternative ASNase or another metabolic pathway utilizing asn-N was expressed in the transformants. One possible route is through activity of an aminotransferase which is functional in mature leaves of white lupin (Atkins et al. 1983). Two genes encoding ASNase, one K^+ independent and a second K^+ dependent, have been recognized in a range of plants, including seeds of lupin species, with overlapping spatial and temporal patterns of expression (Bruneau et al. 2006). While it is likely that the RT PCR primers used would have detected transcript for both ASNases, unfortunately seeds of these transgenic plants were not assessed for expression of alternative metabolic pathways for asn utilization.

There have been a number of suggestions (reviewed in Castaings et al. 2011) that the N status of a plant and its nutritional requirement for N are "sensed and signaled" through the action of translocated N solutes, and a recent review has implicated asn accumulation in legumes (Sulieman and Tran 2012) with such a role. The review has documented many instances that lead to high levels of asn in a range of tissues and exudates from both xylem and phloem. Sulieman et al. (2010) analyzed N solutes collected in an EDTA solution from leaves of *Medicago truncatula*, and under conditions of low Pi supply to the plant, the amount of amino acids collected increased fivefold due almost entirely to a 20-fold increase in asn. The level of asn accumulating in nodules under Pi limitation increased markedly and nitrogenase activity, assayed as H_2 evolution, decreased some 50 % (Sulieman et al. 2010).

These data have led to the proposal that phloem-borne asn may regulate nitrogenase activity either directly or indirectly through the transport of the amide or its metabolites across the symbiosome membrane or through regulation of O_2 diffusion (Atkins and Minchin 1995). Whether other N solutes are likely to also function in this way, not only in nodules but in other tissues, remains to be determined. Similarly, a regulatory role has been linked to sugars and to the idea that phloem is also involved in systemic signaling carbohydrate status. While for many years the likely "signals" were essentially confined to the well-documented group of plant growth regulators or hormones, and these have been recovered from both xylem and phloem, more recently a large number of macromolecules have been described in phloem exudates, including exudates collected from aphid stylets (reviewed in Atkins et al. 2011). While, for many of these, a signaling role, and even their translocation, is yet to be established, the realization that phloem is not simply a conduit supplying the nutritional ingredients for growth opens a new and exciting perspective for understanding how the components of a plant communicate.

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Chapter 7
Plasmodesmata and Phloem-Based Trafficking of Macromolecules

Dhinesh Kumar, Ritesh Kumar, Tae Kyung Hyun, and Jae-Yean Kim

Abstract Plant cell fate specification, responses to stimuli, and developmental coordination at multicellular level are achieved by cell-to-cell communication. Plasmodesmata (PD), cytoplasmic nanochannels interconnecting neighboring cells, sophisticate such communication by regulating exchange of molecules. The composition, organization, and architecture of these interconnecting channels have emerged, and several models are available. PD play as major gatekeepers of signaling macromolecules such as proteins and/or RNAs and establish domains of symplasmically connected cells either to facilitate or restrict the transport of such signaling molecules. This chapter is dedicated for those who seek insightful review through supporting evidence on intercellular trafficking of a range of endogenous proteins and their updated non-cell-autonomous protein pathway (NCAPP) machinery/components taking care, together, of plant development.

Keywords Intercellular trafficking • Symplasmic communication • Plasmodesmata • Phloem

Abbreviations

CC	Companion cell
CmPP1 CmPP2, CmPP16, and CmPP36	*Cucurbita maxima* phloem protein 1, 2, 16, and 36
CPC	CAPRICE

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FLO	FLORICAULA
FT	FLOWERING LOCUS T
GFP	Green fluorescent protein
KN1	KNOTTED1
LFY	LEAFY
NCAP	Non-cell-autonomous protein
NCAPP	Non-cell-autonomous protein pathway
PD	Plasmodesmata
RNA	Ribonucleic acid
SE	Sieve element
SHR	SHORT-ROOT
STM	SHOOT MERISTEMLESS
UPB1	UPBEAT1

7.1 Introduction

Plant cells do not, physically, exist in isolation and must constantly take up information from, and react to, their environment. Cells accomplish this by translating signals that they receive from their environment and/or from adjacent cells to an appropriate reaction inside the cell (Fig. 7.1). There are increasing evidences that specific macromolecular signals such as proteins and RNAs are involved in cell-to-cell communication in plants (Casson and Lindsey 2003; Doerner 2003). In one mechanism, a protein ligand secreted by a cell migrates within the cell walls. After reaching the surface of the target cell, the ligand interacts with the plasma-membrane-bound receptors to regulate differential gene expression (Fig. 7.1); CLAVATA-mediated signaling falls into this class of apoplastic pathway which regulates the stem cell fraternity in the shoot apical meristem (SAM) of *Arabidopsis* (Doerner 2003). In plants, the exchange of such signals is also sophisticated by plasmodesmata (PD), symplasmic intercellular membrane-lined nanochannels adjoining two cells (Fig. 7.1). PD mediate not only the flux of small solutes but also cell-to-cell transport of proteins, RNAs, and RNA/protein complexes to orchestrate growth, development, and defense processes at the tissue/whole-organism level (Lucas 1995; Haywood et al. 2002; Roberts and Oparka 2003). Over the past years, molecular analyses of PD have been increased and led to a complete change in scientific understandings of which the development of PD is one of the most crucial events in the evolution of higher plants (Karol et al. 2001). The composition, organization, and architecture of these interconnecting channels have been being emerged, and several models are available. While the plasma membrane (PM) delimits the cytoplasmic sleeve within PD, and the appressed endoplasmic reticulum (ER) strand and proteinaceous components occupy the sleeve, this whole structure adds intricate structural and mechanistic elements to each channel (Robards and Lucas 1990; Maule 2008). Recently, such a complicated structure has been foreseen as a supramolecular communication network making it invaluable in plant

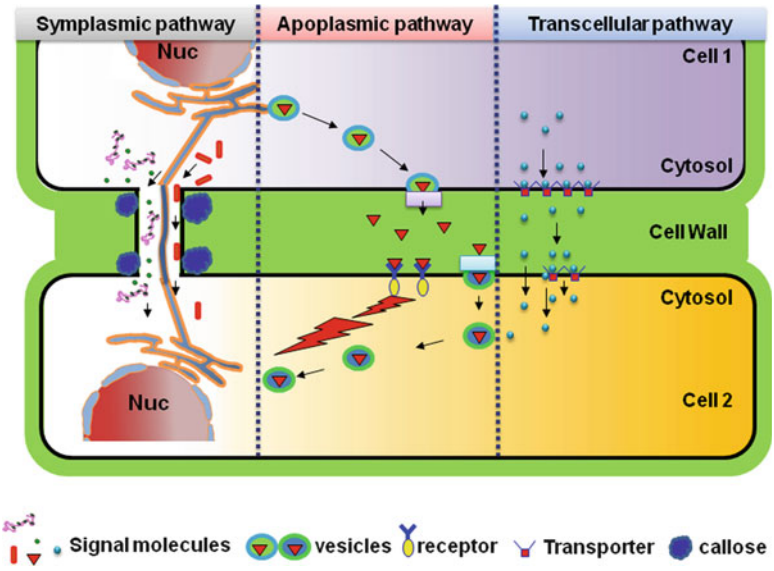

Fig. 7.1 Schematic representation for the cell-to-cell signaling pathways in plants. Symplasmic pathway involves direct intercellular symplasmic transport of signaling molecules through plant-specific intercellular tunnels, plasmodesmata (PD). These molecules range from ions and small molecules to non-cell-autonomous transcription factors and RNAs. Apoplasmic pathway is characterized by secretion of signaling molecules and perception by receptors in neighboring cells as shown in the case of plant hormone perception and transport through transports. Transcellular pathway is characterized through secretion or export and internalization or import as shown in polar auxin transport

developmental plasticity by uncovering several mechanisms, which include selective pathways characterized by the capacity of PD to get dilated by the combinatorial action between its components and molecules on move and diffusion-based nonselective pathway (Lucas and Lee 2004; Zambryski 2004; Liarzi and Epel 2005; Christensen et al. 2009). In both cases, the ultimate response is to establish domains of symplasmically connected cells either to facilitate such transport as seen in the case of meristem maintenance or to restrict it as seen in the case of sink/source transition during leaf development (Lucas 1995; Oparka et al. 1999).While the mechanistic details of how such a range of phenotypic plasticity is controlled by PD are not well understood, recent advances have increasingly focused on plasmodesmal trafficking at a molecular scale either to localize and decipher a role for an individual PD component or to characterize the functional feature of a trafficking protein, and both, thereby strengthening the long-lasting notion that PD play as major gatekeepers of signaling molecules. By keeping this in mind, this chapter is dedicated for those who seek insightful review by supporting evidences on intercellular trafficking of a range of endogenous proteins and their updated non-cell-autonomous protein pathway (NCAPP) machinery/components taking care, together, of plant development.

7.2 Cell-to-Cell Trafficking of Plant Transcription Factors and Their Signaling Mechanisms

Over the past years, molecular analyses of PD and its structure have been increased and have, therefrom, led to a complete change in scientific understanding that the development of PD is one of the most crucial events in the evolution of higher plants (Karol et al. 2001). PD provide a symplasmic intercellular trafficking pathway for non-cell-autonomous macromolecules including TFs and diverse RNAs. Intercellular RNA and RNAi spread have been well described in other recent reviews (Chitwood and Timmermans 2010; Hyun et al. 2011; Brosnan and Voinnet 2011), thus we focus henceforth on the cell-to-cell movement of TFs. The TF movement through PD is convincingly divided into two mechanisms that can either be by nonselective movement, which was first proposed for LEAFY (LFY) protein movement among adjoining cells, or can be by selective trafficking, which involves specific interaction between non-cell-autonomous protein (NCAP) and the components of non-cell-autonomous protein pathway (NCAPP) as provided by the studies on the first endogenous plant TF, KNOTTED1 (KN1), required for maize leaf and shoot meristem development (Lucas et al. 1995; Kim et al. 2005). PD regulation of regulatory TFs plays a central role for the control of global developmental program, ranging from the formation of embryonic root to induction of flowering (see Table 7.1). In this section, it is reviewed on the testimonial findings for intercellular trafficking of TFs and discussed on the likely mechanisms that can mediate these macromolecular movements from cell-to-cell (also see Table 7.2).

7.2.1 KNOX-Family Homeodomain TFs

The first endogenous protein model shown to traffic cell-to-cell was the maize homeodomain (HD) protein, KN1 (Lucas et al. 1995), which got name from dominant gain-of-function alleles that resulted in abnormal knots over the lateral veins of maize leaves due to ectopic cell divisions (Hake and Freeling 1986; Sinha and Hake 1990). Mosaic analysis of a dominant *Kn1* allele came up with striking evidence that it acts non-autonomously during maize leaf development (Hake and Freeling 1986). The authors interpreted the results of mosaics showing tissue layer-specific *Kn1* expression pattern in such a way that dominant mutant allele present in inner mesophyll and vascular cells was enough to generate knots, although this phenotype is the result of ectopic divisions in all layers, and, thus, concluded that *Kn1* functioning in cell fate and division is non-autonomous to the layers it was not originally expressed. *KN1* gene is expressed in the shoot apical meristem (SAM) that is the self-renewing structures consisting of stem cells and their immediate daughters (Smith et al. 1992). An endogenous role for KN1 and for an *Arabidopsis* homolog SHOOT MERISTEMLESS (STM) in the initiation and/or maintenance of the SAM was determined from the studies on

Table 7.1 Non-cell-autonomous proteins and their characteristics

Proteins	Function(s)	Tissue/localization	Reference(s)
KNOTTED1	Initiation and/or maintenance of the SAM	Shoot apex/nucleus	Lucas et al. (1995), Kim et al. (2003), Jackson (2005)
SHOOT MERISTEMLESS (STM)	Initiation and/or maintenance of the SAM	Shoot apex/nucleus	Sinha et al. (1993), Long et al. (1996)
AtKNAT1/BP	Regulation of inflorescence architecture	Inflorescence SAM/ nucleus	Douglas et al. (2002), Venglat et al. (2002)
FLORICAULA (FLO)	Floral meristem identity	Floral meristem/nucleus	Hantke et al. (1995)
LEAFY	Floral meristem identity	Floral meristem/nucleus	Sessions et al. (2000), Wu et al. (2003)
DEFICIENS (DEF)	Floral organ development	Shoot apex	Sommer et al. (1990),
GLOBOSA (GLO)	Floral organ development	Shoot apex	Schwarz-Sommer et al. (1992)
CAPRICE (CPC)	Root hair differentiation	Root epidermis hair cell/ nucleus	Kurata et al. (2005)
SHORT-ROOT (SHR)	Root endodermal layer identity	Stele/nucleus	Helariutta et al. (2000), Nakajima et al. (2001)
UPBEAT1 (UPB1)	Root development	Vascular tissue of elongation and maturation zones, cells of LRC	Tsukagoshi et al. (2010)
FLOWERING LOCUS T (FT)	Long-day-dependent flowering	Leaf phloem companion cells	Kardailsky et al. (1999), An et al. (2004)
CmPP16	RNA transport	Phloem/cytosol-nucleus	Xoconostle-Cazares et al. (1999)
CmHSC70	HSC70 chaperone	Phloem/cytosol	Aoki et al. (2002)
CmRBP50	RNA transport	Phloem/cytosol-nucleus	Ham et al. (2009)
CmPP36	Cytochrome b5 reductase	Phloem/cytosol	Xoconostle-Cazares et al. (2000)
CmPP1 and CmPP2	Interact with PD to increase SEL and to mediate their own transport	Phloem/filaments	Balachandran et al. (1997)

loss-of-function mutations on *kn1* and *stm*, leading to a loss of indeterminacy in the SAM premature termination of shoot development (Sinha et al. 1993; Long et al. 1996). Later, *in situ* hybridization and immunolocalization experiments demonstrated the fate of KN1 being itself a cell-to-cell signal to foster abnormal cell

Table 7.2 Factors and/or regulatory mechanisms influencing intercellular movement in higher plants

Factors	Proposed/proved mechanisms	Plant system/tissue studied	Reference(s)
Callose-related factors			
Reactive oxygen species (ROS)	Accumulates callose by maintaining redox homeostasis in plant cells	ROS increase during aging stimulates PD callose, thereby inhibiting phloem transport in castor bean; restricted movement of soluble GFP from phloem CC into root meristem of *Arabidopsis*	Jongebloed et al. (2004), Benitez-Alfonso et al. (2009)
Aluminum (Al)	Al may induce callose at neck regions of PD	Al-induced root growth inhibition in wheat was related with Al-induced callose deposition at PD, thereby blockage of symplasmic transport	Sivaguru et al. (2000)
Calcium ions (Ca2+)	Increasing intracellular calcium level through ionophore (IP3) may signal activation of 1–3-β-glucan synthase located in PM at PD	Physiological elevations in cytoplasmic free Ca^{2+} by cold or ion injection result in transient closure of maize PD	Kauss, (1985), Holdaway-Clarke et al. (2000)
ATP	Lack of ATP may perturb dynamic balance between callose deposition and degradation at PD channels so as to bring about a reduction in callose and thus an increase in SEL	PD-mediated cell-to-cell transport in wheat roots is modulated by ATP-anaerobic stress	Wolf et al. (1989), Cleland et al. (1994)
ISE1 and ISE2	Defect in these mitochondrial and chloroplast helicases leads to increased PD SEL via differential redox states	Altered PD biogenesis in ise1 and ise2 embryos of *Arabidopsis* is likely via alteration of genes encoding PD-localized and/or callose-regulating products, thereby altering PD transport	Burch-Smith et al. (2011), Stonebloom et al. (2012)
DSE1	A WD-repeat protein critical for plant morphogenesis and reproduction, dse1 mutant ovules accumulate callose as internal aggregates	*Arabidopsis* dse1 embryos showed reduced PD-mediated dye movement; silenced DSE1 in tobacco also lead to restricted TMV:MP-GFP movement	Xu et al. (2012)

m-Type thioredoxin/ GFP-arrested trafficking 1 (TRX-m3/GFP1)	gat1 mutation results in an accumulation of ROS and thereby increases PD callose	*gat1* affects symplasmic permeability that is essential for meristem maintenance in *Arabidopsis*	Benitez-Alfonso et al. (2009)
Chaperone activity			
CmHSC1–2	Unfolding of NCAPs	Chaperone machinery to regulate both protein and RNA entry, long-distance movement, and exit from the phloem	Aoki et al. (2002)
Chaperonin-containing TCP1 (CCT8)	Refolding of NCAPs and viral MPs in receiver cells	Post-translocational refolding of KN1/STM to maintain *Arabidopsis* stem cell homeostasis	Xu et al. (2011)
Other factors			
NtNCAPP1	A specific PD receptor protein recognizes and shuttles NCAPs to PD	Tobacco leaf and floral developmental defects in silenced NtNCAPP1 plants	Lee et al. (2003)
Viral MP-binding protein 2C (MPB2C)	Regulate entry of viral MPs and HD proteins into the NCAP pathway	Negatively regulate KN1 association to PD and so its transport in tobacco	Kragler et al. (2003), Winter et al. (2007)
Synaptotagmin (SYTA)	An endosomal-membrane trafficking protein may influence movement of intercellular proteins	Movement of CaLCuV-MP and TMV-MP is delayed or inhibited in *Arabidopsis* defective in SYTA	Lewis and Lazarowitz (2010)
MONOPTEROS7 (MP)	A novel MP-dependent intercellular signal in embryonic root specification	MP mediates intercellular movement of TMO7 in embryo to hypophysis during *Arabidopsis* embryogenesis	Schlereth et al. (2010)
SHORT-ROOT INTERACTING EMBRYONIC LETHAL (SIEL)	ARM and HEAT domains containing protein that interacts with SHR and other NCAPs to facilitate their intercellular movement	Hypomorphic mutant phenotypes suggest involvement of SIEL in *Arabidopsis* root patterning	Koizumi et al. (2011)

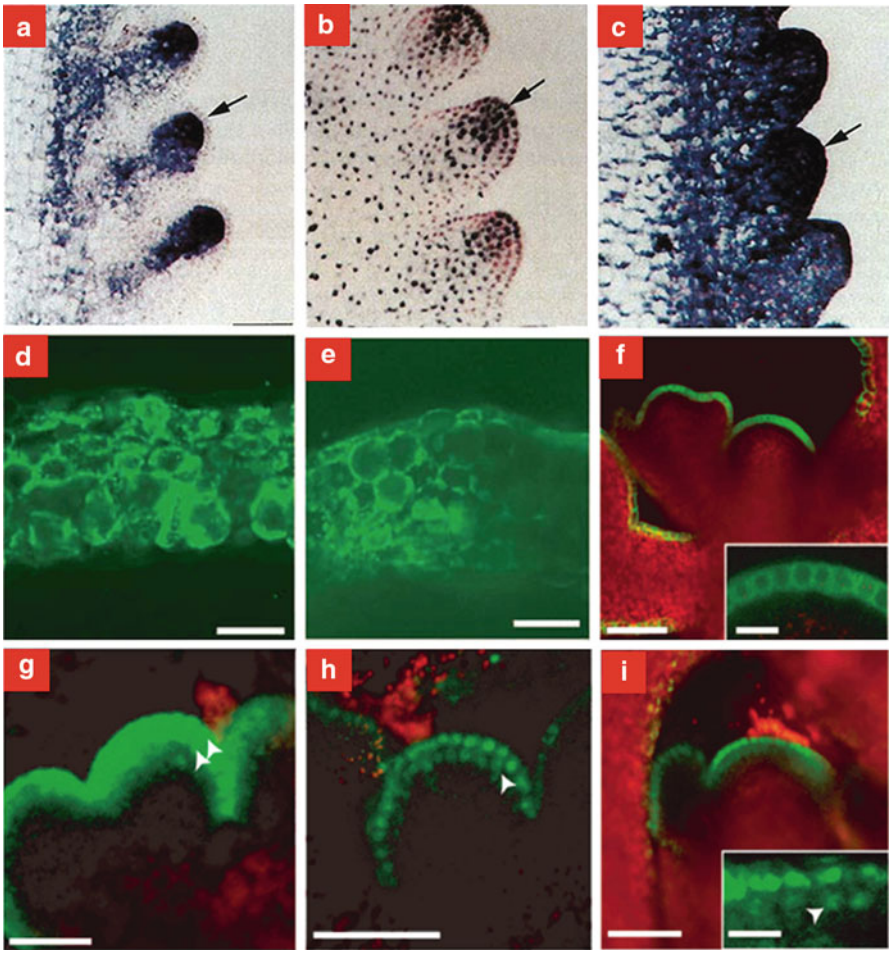

Fig. 7.2 KNOX-family proteins act non-cell autonomously. (**a**)–(**c**) KN1 protein and mRNA localization differs in meristems. (**a**) *In situ* localization of *KN1* mRNA through three spikelet meristems on maize ear inflorescence strongly stained in the L2 layer, but not stained in L1 layer (*arrow*). But KN1 protein localization strongly labeled both L2 and L1 layers of the meristem (*arrow*) (**b**). As a control, ubiquitin mRNA expression was shown in all cells, including those of L1 (*arrow*) (**c**). (**d**) and (**e**) Confocal images of leaf cross-section of transgenic *Arabidopsis* expressing cell-autonomous GFP (**d**) and GFP~KN1 fusion (**e**) under the control of mesophyll-specific promoter. A bright fluorescence for cell-autonomous GFP is seen only in mesophyll tissue, but not in epidermal pavement cells (**d**), and the same is seen in all epidermal and subepidermal cells (**e**). (**f**)–(**i**) Confocal images of inflorescence SAM of transgenic *Arabidopsis* expressing cell-autonomous GUS~GFP (**f**) and GFP fusions to KN1 (**g**), STM (**h**), and KNAT1 (**i**) under the control of L1 layer-specific promoter. Cytoplasmic fluorescence is clear only in L1 layer for cell-autonomous GFP (**f**; higher magnification in inset), but GFP fusions to KNAT1, STM, and KN1 showed strong L1, L2, and weaker L3 green fluorescence. Movement is pointed out by *white arrowheads* (**g**, **h**, and **i** [Inset]) (Parts **a**–**c** from Jackson et al. (1994), available at http://dev.biologists.org/content/120/2/405.abstract?ijkey=20477655b76c0cd78cf032cf8722764ecc9286ff&keytype2=tf_ipsecsha; and parts **d**–**i** from Kim et al. (2003), available at: http://dev.biologists.org/content/130/18/4351.full?sid=c9cfe785-ded2-45cd-b895-c26f66a63b20. Reproduced with permission from The Company of Biologists Ltd.)

division and cell fate in *Kn1* leaves, suggesting, together with other findings, that KN1 protein is detected outside the domain of mRNA expression, implicated the possibility of KN1 trafficking (Fig. 7.2; Smith et al. 1992; Jackson et al. 1994). In maize or tobacco leaves, microinjection experiments of fluorescently cross-linked KN1 protein showed the ability of KN1 to traffic between mesophyll cells, to facilitate the intercellular movement of cell-autonomous dextran probe, and to specifically transport its mRNA, presumably by gating or transiently increasing PD SEL (Lucas et al. 1995). In support of this notion, the authors revealed a motif, by alanine scanning mutagenesis, at the N-terminus of the homeodomain which was required for KN1 trafficking and for PD gating, since a mutant KN1 (M6) was unable to traffic, undoubtedly suggesting an selective transport of KN1. In continuation to unravel the signaling mechanism of KN1 in a cell into which it traffics, Kim et al. (2002) showed that a GFP-tagged KN1 fusion expressed under specific tissue layers (L1 or L3) in *Arabidopsis* is able to traffic in shoot apical meristem (SAM). Consistent with these results, its L1- or L3-specific expression was able to complement strong *stm* mutants (Kim et al. 2003). Interestingly, intercellular KN1 trafficking was unidirectional in leaf tissues. In both young and mature leaves, GFP-KN1 could not traffic from epidermis to mesophyll, although the reverse trafficking took place. Among the four class I KN1-related homeobox (KNOX) genes encoded by *Arabidopsis* (Bharathan et al. 1999; Reiser et al. 2000), KNOTTED1-like HOMEOBOX PROTEIN 1/BREVIPEDICELLUS (KNAT1/BP) and STM are the most closely related to KN1 and have been shown to traffic, like KN1, in inflorescence SAM (Fig. 7.2; Kim et al. 2003). While STM and KN1 play as a developmental cue in SAM initiation and/or maintenance, KNAT1/BP is involved in the regulation of inflorescence architecture (Douglas et al. 2002; Venglat et al. 2002). Why should KN1 and STM be trafficked between the layers of meristem? Though the question seems to have found an answer from the findings on maize, in *Arabidopsis* it is yet to be exemplified since *STM* mRNA normally accumulates in all of the three layers of SAM (Long et al. 1996). In the case that *KN1* mRNA is undetectable in the maize L1 layer, but protein is clearly present there, it is conceivable that the growth of the varied cell layers in the SAM is coordinated by the trafficking of KN1 from L2 to L1. An independent proof that KN1 functions as a selective NCAP was thrown by Kim et al. (2005) by developing an ingenious trichome rescue assay. In that study, subepidermal expression of a GLABRA1 (GL1)-KN1 fusion protein, but not GL1 itself, in a trichome-deficient *glabra1* (*gl1*) background line restored trichome development in the epidermal layer. This trichome rescue system confirmed once again the capacity of KN1 to mediate cell-to-cell trafficking of its own mRNA and also showed that the HD motif is necessary for selective cell-to-cell trafficking. KN1/STM HD interacts with the MT-associated and viral MP-binding protein, MPB2C, from *Nicotiana tabacum*, and its homolog in *Arabidopsis*, AtMPB2C (Winter et al. 2007). These proteins negatively regulate KN1 association to PD and, consequently, cell-to-cell transport (Winter et al. 2007). The role played by MPB2C proteins in regulating the cell-to-cell trafficking of homeodomain proteins and TMV-MP (Kragler et al. 2003) in plants has advanced the hypothesis that plants use a common pathway involving

MPB2C to regulate the entry of viral MPs and HD proteins into the NCAP pathway. Earlier, it was shown that modifying KN1 size by covalently cross-linking to 6 and 15 nm gold particles exerts steric hindrance during the KN1 translocation process, indicating that a chaperone activity is required for KN1 trafficking (Kragler et al. 1998b). This viewpoint that tertiary protein structure regulate KN1 protein movement, likely through unfolding during the translocation process (Kragler et al. 1998a), was supported by the identification of a class II chaperonin subunit, CCT8 (chaperonin-containing TCP1), through a genetic mutant screen (Xu et al. 2011). An EMS-mutagenized *cct8-1* plant showed a limited movement of KN1/STM most likely due to a loss of post-translocational refolding of the fusion protein by *cct8-1* mutation (Xu et al. 2011). Direct involvement of the chaperonin complex in mediating cell-to-cell signaling by *Arabidopsis* and maize KNOX proteins suggests that this intercellular pathway mechanism is conserved in diverse plant species. More recently, Fichtenbauer et al. (2012) extended the role played by CCT8 in intercellular trafficking of viruses owing to the previous findings that KN1 move from cell-to-cell in a similar fashion as TMV-MPs (Kragler et al. 2000). In this study, the author showed that spread of tobamovirus infection is reduced in *cct8* mutant, suggesting that similar to KN1, viral MPs undergo a change in tertiary structure by unfolding and refolding during their transport via PD to be functionally active in the receiving cells. Although it is accepted that KN1 moves through a selective non-cell-autonomous machinery, it is just a beginning to unravel the components involved in this pathway.

7.2.2 TFs Involved in Floral Development

The SAM undergoes a transition from a vegetative meristem, which initiates leaves, to an inflorescence meristem which initiates floral meristems from its flanks (Weigel et al. 1992). The development of floral identity requires FLORICAULA (FLO) or LEAFY (LFY), which are the members of a plant-specific TF family, to regulate downstream floral homeotic genes (Parcy et al. 1998). Mutation in *FLO* or *LFY* resulted in the inflorescence transition, followed by the altered arrangement of bract or leaf-like organs in a floral phyllotaxy. FLO in *Antirrhinum* is expressed transiently in early flower primordia, and inactivation of the *FLO* gene causes the transformation of flowers into inflorescence shoots (Carpenter and Coen 1995). The transposon-induced mutational analysis of periclinal chimeras and *in situ* hybridization revealed that *FLO* functions in non-cell-autonomous manner based on the demonstration that irrespective of the cell layer in which *FLO* is expressed, floral meristem identity was restored (Carpenter and Coen 1995; Hantke et al. 1995). In all kind of periclinal chimeras, the expression of downstream target genes, *DEF* and *PLENA* (*PLE*), was identified despite *FLO* being expressed only in a single layer, indicating a non-cell-autonomous function of FLO between cell layers in the floral meristem to activate downstream transcriptional target genes (Hantke et al. 1995). The nature of the cell-to-cell signaling in floral meristematic layers was evidently

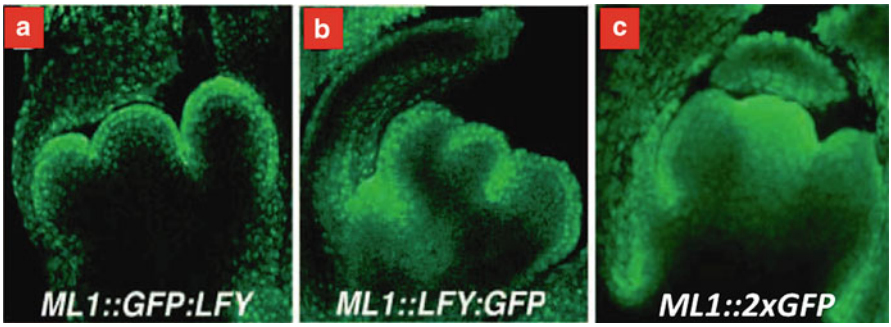

Fig. 7.3 Intercellular movement of LEAFY. (**a**)–(**c**) Confocal images of inflorescence apices of *Arabidopsis* transgenic plants expressing *GFP-LFY*, *LFY-GFP* fusions, and a dimerized *GFP* (*2×GFP*) under the control of L1 layer-specific ML1 promoter. In both the cases of LFY fusions, GFP fluorescence representing nucleus and cytoplasmic spots is seen in several layers into the underlying tissue from the L1 in the apex (**a**), though LFY-GFP showed longer moving range. A gradient of GFP fluorescence in all cell layers with the highest levels in the L1 for 2×GFP is the result of a nontargeted diffusion of 54 kDa-dimerized GFP that is approximately equal to the size of LFY (47 kDa), confirming the diffusion-based intercellular LFY movement (**c**) (Parts **a**–**c** from Wu et al. (2003), available at http://dev.biologists.org/content/130/16/3735.full?sid=08bab89f-ae91-48bb-9c27-8d02ac487ed1. Reproduced with permission from The Company of Biologists Ltd.)

described by Sessions et al. (2000) by showing the movement of LFY, an *Arabidopsis* ortholog of FLO. Like FLO, LFY is also required for the transition from vegetative to reproductive development. Both the mRNA and protein of LFY are normally present in all the three layers of the floral primordia (L1, L2, and L3). However, when *LFY* is expressed in L1 layer (epidermis) using the tissue-specific ML1 promoter in *lfy-12* mutant background, the protein is able to move into the L2 and L3 layers and to rescue the *lfy-12* phenotype (Sessions et al. 2000). Further support for the non-cell-autonomous trafficking mechanism of LFY was obtained from the study by Wu et al. (2003) using functional GFP fusions with LFY expressed under the control of ML1 promoter (Fig. 7.3). LFY moves more readily from the L1 into deeper cell layers than laterally into adjacent, clonally related cells. In addition, comparison of the dynamics of LFY-GFP fusion proteins with 2×GFP and MP-GFP fusion proteins expressed by the same promoter was able to conclude that the LFY movement in floral meristem is driven most probably by diffusion (Fig. 7.3). To be compatible with the conclusion that LFY movement is based on the nonselective diffusion, the authors addressed this through deletion experiments that failed to identify a specific movement signal in LFY, putting forward a possibility of either not having a PD-targeting sequences or the presence of multiple targeting sequences in LFY. Taken together, the evidences presented so far with respect to LFY and FLO foster the testable hypotheses: first, NCAPs can appear to move between cells in a nonselective manner, although there is a need to develop a profound study to establish this LFY-based passive diffusion to be generalized by including other proteins,

other tissues, and other species. Second, at least in meristem, intercellular protein movement can be a default fate if proteins carrying a smaller size than PD SEL are not either expeditiously targeted to specific subcellular locations or retained through formation of multi-protein complexes. In angiosperms, complexity of the different types of organs requires coordination between the three cell layers of meristem suggesting the transmission of signals between layers. The investigation on the nature of the signal and the mechanism they follow to achieve this signaling during plant development has come up with the promising evidences by the involvement of MADS-box proteins as homeotic regulators of floral organ development. In *Antirrhinum*, petal and stamen organ identity was shown to be controlled by the interaction of the flower *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) genes encoding MADS-box TFs (Schwarz-Sommer et al. 1992; Sommer et al. 1990). The mutations on these genes resulted in a homeotic transformation of petals to sepaloid organs and stamens to carpelloid organs. The contribution of DEF and GLO proteins to the control of cell and organ identity has been studied in somatically stable *def* and *glo* periclinal chimeras, obtained using genetically unstable transposon-induced alleles (Carpenter and Coen 1990; Perbal et al. 1996). *In situ* immunolocalization studies showed that a control over the organ identity by DEF or GLO takes place in a non-cell-autonomous fashion. For example, DEF behaved to be autonomous when it was expressed in L1 epidermal layer since no proteins were detectable in underlying L2 and L3 layers, but the same behaved to be non-cell autonomous when expressed in L2 and L3 (Perbal et al. 1996), providing an evidence that polar trafficking of DEF protein is likely to be controlled by cell-type-specific directional control system as seen in the case of KN1 (Kim et al. 2003). Most of the findings made with DEF and GLO have been confirmed for their *Arabidopsis* orthologs, APETALA3 (AP3), and PISTILLATA (PI), despite being different in their expression patterns (Riechmann and Meyerowitz 1997). PI can non-cell autonomously control petal and stamen development from the epidermis, a situation contrast to that in *Antirrhinum,* as revealed by the wild-type phenotype of X-ray-induced chimeras (Bouhidel and Irish 1996). AP3 and PI differ in the extent of their cell-autonomous and non-cell-autonomous contributions to *Arabidopsis* organ identity. AP3 in L1 has a little non-cell-autonomous effect on organ shape, and in stamens to control epidermal cell identity, according to the earlier observations in genetically engineered *Arabidopsis* plants raised to mimic somatically stable transposon-induced alleles (Jenik and Irish 2001). Because of these species-specific differences in signaling, and in cell-to-cell movement of MADS-TFs, Efremova et al. (2001) addressed this issue by expressing *DEF* or *GLO* using an endodermal-specific promoter in both *Antirrhinum* and *Arabidopsis*. Interestingly, expression of *DEF* and *GLO* in the L1 of *Arabidopsis* plants carrying a mutation in APETALA3 (the DEF ortholog) restored normal floral organ development, indicating a much greater degree of non-cell autonomy. Thus, in *Arabidopsis*, the putative regulatory elements involved in cell-to-cell trafficking of NCAPs may not impose effective control over DEF/GLO movement, undoubtedly drawing a plot over the different degrees of autonomy that appears to be a specific characteristic of a species rather than of the orthologous proteins. Clearly, there is a pressing message to advance our understanding that the signaling by the

movement of MADS-box proteins between cell layers is an alternative pathway among several other mechanisms dedicated to orchestrate the floral organ development.

7.2.3 TFs Involved in Epidermal Cell Patterning

The epidermis has become a well-studied model for cell differentiation and cell patterning in plants (Larkin et al. 2003). A comparison of the underlying patterning mechanisms during the development of three epidermal cell types, trichomes, root hairs, and stomata has advanced our understanding on cell fate determination: for instance, trichome development and root epidermal patterning involve a common mechanism comprising of closely related cell fate transcription factors and a similar lateral inhibition signaling pathway. Cell-to-cell communication events that are crucial for establishing the position-dependent pattern of root epidermal cells have got much attention by correlating between cell position and cell type differentiation. A positive regulation exerted by *CAPRICE* (*CPC*), a gene encoding a small protein of 94 amino acid residues with a Myb-like DNA-binding domain, was shown to specify the hair cells by suppressing the expression of a negative regulator for hair cell specification, GLABRA2 (GL2), suggesting an upstream role for CPC in root hair (RH) cell patterning (Wada et al. 1997). These findings were consistent with the hairless phenotype of loss-of-function *cpc* mutants (Wada et al. 1997) and that of *gl2* showing a hairy root (Masucci et al. 1996). Since CPC is a positive regulator in RH patterning, its expression site was expected to be hair cells, but it was surprisingly found in non-hair (NH) cells (Wada et al. 2002). Interestingly, transgenic plants expressing a GFP fusion of CPC driven by its own promoter, *pCPC*, showed the fluorescence in the nuclei of both NH and RH cell files, plotted down a model in which CPC traffics from hairless to hair cells where it could likely repress *GL2* expression to execute RH cell file (Fig. 7.4; Lee and Schiefelbein 2002). To elucidate the mechanisms of cell-to-cell movement of CPC, an elegant research carried out by Kurata et al. (2005) came up with several insights into intercellular movement of CPC. By deletion mutagenesis on CPC-GFP, it was shown that two motifs, one in the N-terminal region and the other in the Myb domain, are responsible for the cell-to-cell movement of CPC, especially amino acids tryptophan (W76) and methionine (M78) in the Myb domain. Moreover, it was also demonstrated that the movement of CPC is tissue specific as when CPC is expressed in stele, it did not seem to be moved. A notion that CPC selectively increases the PD SEL is supported by the evidence that when tandem 3×GFP was expressed under *pCPC*, it was unable to move out of the tissue it was expressed; but when CPC was fused to 3×GFP, fusion protein could be detected in all epidermal cells, conferring a gain-of-trafficking activity to 3×GFP (Kurata et al. 2005). Non-cell autonomy of ETC1, ETC2, ETC3 (ENHANCER OF TRY AND CPC1, CPC2, and CPC3), TRY (TRYPTICHON), and CPC, the MYB family TFs, was shown to pattern trichomeless cells on *Arabidopsis* leaves through binding to the GLABRA3 (GL3)/EGL3

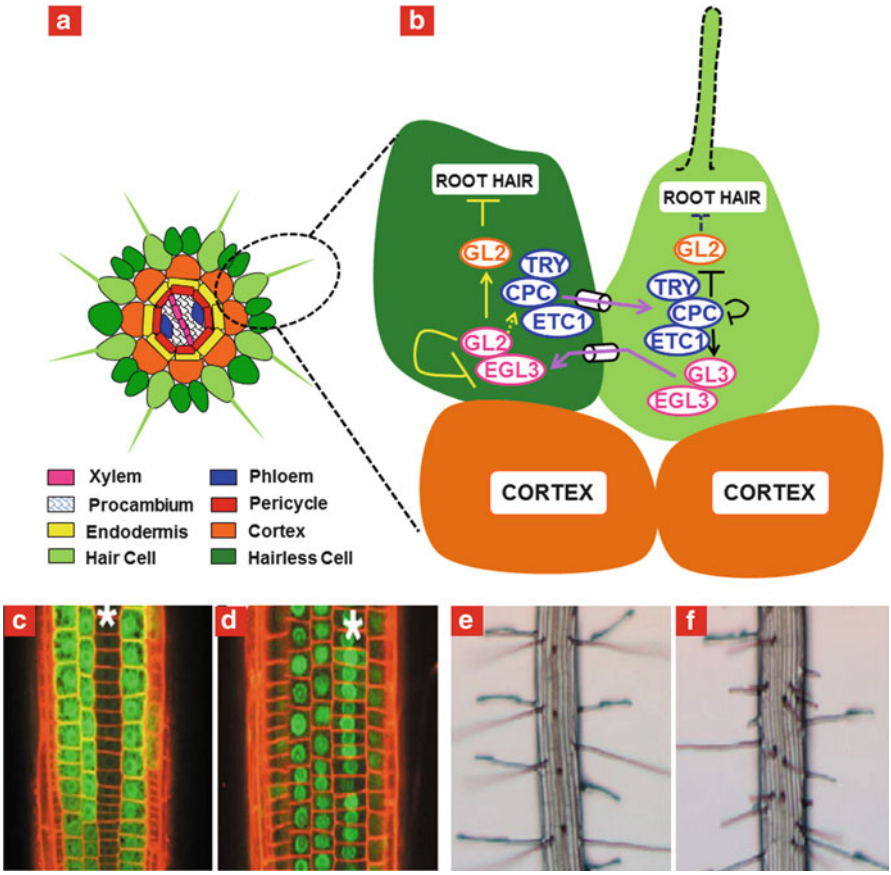

Fig. 7.4 Intercellular movement of proteins in root epidermal patterning in *Arabidopsis*. (**a**) Schematic cross-section of an *Arabidopsis* root showing alternating cell files with root hair outgrowths. (**b**) A simplified model of intercellular regulatory network in root hair cell patterning involving mutual trafficking of CPC/TRY/ETC1 and GL3/EGL3. CPC and/or other Myb proteins move from non-hair cell to hair cell to specify hair cell identity either by activating GL3/EGL3 or repressing GL2 expression. Opposite to CPC, GL3/EGL3 moves from hair cells to hairless cells to specify non-hair cell identity. Also, the specified epidermal identities are maintained by CPC- and GL3-mediated autoinhibitory activity on their own transcription. (**c**) and (**d**) Confocal images of transgenic *Arabidopsis* expressing *2×GFP* (**c**) and *CPC-GFP* fusion (**d**) driven by *pCPC*. GFP fluorescence for CPC-GFP protein is seen in all cell files, possibly due to the trafficking of CPC-GFP into hair cells (*asterisk*). The same for cell-autonomous nuclear GFP is absent in hair-forming cell files (*asterisk*) but detected in all non-hair cell files. (**e**) and (**f**) Root hair phenotype for the plants in (**c**) and (**d**) is different from each other. *TRY* TRYPTICHON, *GL2* GLABRA2, *GL3* GLABRA3, *ETC1* ENHANCER OF TRY AND CPC1, *EGL3* ENHANCER OF GLABRA3 (Parts **c**–**f** from Kurata et al. (2005), available at http://dev.biologists.org/content/132/24/5387.full?sid=65cc1e76-de70-4260-9c87-4c537c6b5276. Reproduced with permission from The Company of Biologists Ltd.)

complex (Bernhardt et al. 2003). Especially, ETC3 is expressed preferentially in incipient trichome cells where it localizes both to the nucleus and the cytoplasm. The protein then moves into the neighboring non-trichome (pavement) cells, where it becomes nuclear localized. The ability of ETC3 and the related protein, CPC, to move between cells in the leaf is affected by the presence of GL3, suggesting that affinity for GL3 may affect mobility of these proteins (Bernhardt et al. 2005). Unlike in the leaf, GL3 traffics between from trichoblasts to atrichoblasts to specify non-hair cell fate of root in the reverse direction of CPC movement, aided to conceive a proposal based on mathematic modeling, which exemplifies that intercellular movement of CPC and GL3 provides a mutual support mechanism regulating root hair patterning (Savage et al. 2008). A combined approach involving genetics and mathematical modeling provides another evidence that TTG1 (TRANSPARENT TESTA GLABRA1) is the only positive regulator of trichome initiation to move nondirectionally in the epidermis and is trapped in the incipient trichome cells presumably via direct interaction with GL3 (Bouyer et al. 2008; Pesch and Hulskamp 2009; Balkunde et al. 2010). Since there have been direct evidences that hair cell and trichome fate are determined by the relative ratio and movement of the TFs (Dolan and Costa 2001; Bernhardt et al. 2005), future researches that would aim in determining the capacity for these TFs to traffic through PD and the identification of putative controlling system potentiating its targeted movement will shed more light into the PD-mediated intercellular communication in plants.

7.2.4 TFs Involved in Root Development

The *Arabidopsis* root has proven to be a very tractable system to dissect the molecular basis of root development, mainly because it has an elegant pattern simplicity of cell organization and stereotyped cell divisions that eventually brings about the various cell types (Fig. 7.5). The longitudinal section of root undergoing embryonic divisions clearly discerns an outermost epidermal, cortical, and endodermal layers surrounding the stele of inner vascular tissues (Dolan et al. 1993). This predetermined pattern of the *Arabidopsis* root by several asymmetric divisions of initial cells is well suitable to study cell fate specification in a position-dependent manner (van den Berg et al. 1995). An era where a number of mutants affecting particular aspects of root patterning was identified and characterized is still seen everlasting. One of such mutants, short-root *(shr)*, perturbs the formation of a cortex and endodermal double layer and places only a single layer of cortex due to the failure in division of cortex/endodermal initials (CEI), marked not only a role for SHR in asymmetric divisions of CEI but also in the endodermal fate specification (Helariutta et al. 2000). SHR was found to be a member of the GRAS family TFs, acting together with another GRAS family TF, SCARECROW (SCR), in normal root cell division and identity. The breakthrough finding that SHR acts in a non-cell-autonomous fashion came from the study, which surprisingly detected *SHR* transcripts only in the stele, but not in the endodermis and CEI (Helariutta et al. 2000).

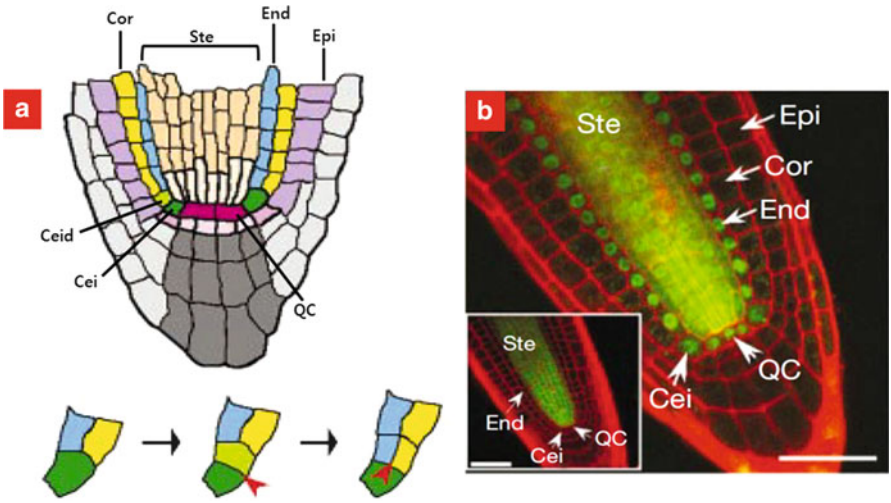

Fig. 7.5 SHR transcription factor movement in root. (**a**) Schematic longitudinal section of an *Arabidopsis* primary root pattern for wild-type seedlings and differentiation of the ground tissue layers as a result of asymmetric division of the cortex/endodermis initial cell (CEI). (**b**) Confocal image of an *Arabidopsis* transgenic seedling expressing *SHR-GFP* under the control of *pSHR* localizes the fusion protein in stele. In endodermal layer GFP fluorescence is predominantly in nuclei. Inset in (**b**) shows cell-autonomous GFP fluorescence only in stele, indicating the stele-specific activity of *pSHR*. *pSHR* SHR promoter promoter, *Ste* stele, *End* endodermal layer, *Cei* cortex/endodermal initials, *Epi* epidermal layer, *QC* quiescent center, *Ceid* daughter of Cei (Parts **a** and **b** reprinted from Nakajima et al. (2001), by permission from Macmillan Publishers Ltd.)

To this unusual speculation of its non-cell autonomy, further experiments have provided supportive and conclusive evidences, of which a fusion of GFP and SHR made under the control of SHR promoter (*pSHR*) was not only able to rescue the *shr* mutant phenotype, but the fusion protein was detected in the stele and the endodermis (Fig. 7.5; Nakajima et al. 2001). Confocal images of *pSHR::SHR:GFP* roots in which GFP fluorescence was detected to be in the nucleus and the cytoplasm of stele, but the same was detected to be only in the nucleus of endodermal layers, were strongly suggestive that SHR traffics from the stele to a single adjacent layer of cells, in which it specifies endodermal cell fate by promoting asymmetrical cell division (Fig. 7.5; Nakajima et al. 2001). To comprehend the role of SHR movement in endodermal cell fate, the transgenic plants were made by expressing SHR directly into the endodermal layer, where it normally moves, using SCR promoter (*pSCR*). The intriguing results of this study are that an increase in cell layers reminiscent to endodermis where SHR protein could also be detected, and a functional *SCR* gene is essential to this superfluous phenotype, indicating an autocatalytic mechanism since SHR protein traffics from where they are expressed to the adjacent layer to activate the *pSCR* and thereby reactivating its own expression and so iterating to specify multiple endodermis (Nakajima et al. 2001). Subsequent studies, therefore,

aimed to unravel the specificity and range of SHR TF movement by addressing the question that putative directional/regulatory system is likely to work in endodermis. According to Sena et al. (2004), the non-cell autonomy of SHR is controlled by cell-specific characteristic because SHR could move out from stele, but not from phloem companion cells (CC) and atrichoblasts of epidermis (Sena et al. 2004). In an another independent research carried out by Gallagher et al. (2004), the tightly regulated movement of SHR was evident when a mutant form of SHR (mutation in T109 amino acid residue), which blocks the nuclear localization and retains the protein only in stele cytoplasm, was even unable to move to adjacent endodermis, suggesting the existence of selective non-cell-autonomous pathway components. Since SCR and SHR were found to interact with each other to regulate the expression of downstream target genes, whose expression goes down in *shr* and *scr* mutants, a study recently highlighted on the role played by SCR in SHR movement. The level of nuclear localization of SHR was directly proportional to the amount of SCR proteins, confirming the involvement of a positive feedback loop, by which SCR together with the trafficked SHR accelerates their own expression. This model explains further that, upon entering into the endodermis, SHR might be sequestered to nucleus by SCR to regulate their own expression above the native level and also downstream gene expressions, keeping SHR always busy in this pathway and thereby blocking SHR movement to other cell layers. This process can be a conserved one among plant kingdom as a single layer of endodermis is present in all plants, and also recently the SHR and SCR proteins were demonstrated to act upon root cell patterning in a similar fashion to their *Arabidopsis* homologues (Cui et al. 2007). Despite the studies related to the regulatory pathways that mediate expression and activity of SHR are continuing to pour (Helariutta et al. 2000; Nakajima et al. 2001; Paquette and Benfey 2005; Levesque et al. 2006; Welch et al. 2007), there is little or no direct evidence concerning the factors that promote its movement. Even, the downstream targets of SHR-SCR complex, JAKDOW (JKD) and MAGPIE (MGP), which encode members of a plant-specific family of zinc-finger proteins, were shown to act in a SHR-dependent feed-forward loop to regulate the range of action of SHR and SCR and appeared to mark their regulatory role in SHR movement by rapidly enhancing the nuclear sequestration of SCR-dependent pathway (Welch et al. 2007). Very recently Koizumi et al. (2011) took the biochemical tool to identify the factors promoting SHR movement, and succeeded in their objective by identifying and characterizing a gene, using yeast two-hybrid screening, SHORT-ROOT INTERACTING EMBRYONIC LETHAL (SIEL), which was found to interact with SHR and has a role in root morphogenesis. *siel*-3 and *siel-4,* the hypomorphic alleles, produce defects in root patterning and reduce SHR movement. Both SHR and SCR regulate expression of SIEL, and SIEL localizes to the nucleus and cytoplasm of root cells where it is associated with endosomes. There are reports on selective transport of various viral movement proteins that not only associate with the ER, Golgi, but also with the recycling endosomes in order to target PD to spread infection. In animal cells, endosomal-associated intracellular trafficking of cargos, for example, ENGRAILED, was required for a secretion-internalization-based intercellular protein trafficking pathway (Joliot et al. 1998).

Therefore, endosomal vesicle-based trafficking might be an alternative pathway for endogenous NCAPs to get transported into their neighboring cells. However, experimental evidences are yet to come. One of the central aspects of development in multicellular organisms is the balance between cellular proliferation and differentiation. A bHLH family of TF, UPBEAT1 (UPB1), has recently been included in the list of factors controlling this balance by showing that UPB1 directly regulates differential expression of peroxidases, which in turn modulate the balance of reactive oxygen species (ROS) between the cell proliferation and cell elongation zones where differentiation begins (Tsukagoshi et al. 2010). It is interesting to note that UPB1 mRNA or UPB1 promoter-driven reporter was expressed in the vascular tissue of the elongation and maturation zones and in cells of the lateral root cap (LRC) close to the transition zone. But, GFP fluorescence for the translational reporter (*pUPB1::UPB1:GFP*) was primarily localized to the nuclei of all cell types in the elongation zone and weakly in the maturation zone; expression of this translational fusion in the *upb1-1* line rescued the mutant phenotype. Such difference between mRNA and protein localization suggested that the UPB1 protein moves from the LRC or vascular tissue to cell files in the elongation zone. This may explain a correlation existing between the position of LRC furthest from the root tip and the transition zone (Willemsen et al. 2008). Non-cell-autonomous effects of an another mobile bHLH-TF, TARGET OF MONOPTEROS7 (TMO7), have been implicated during *Arabidopsis* embryogenesis where an extraembryonic cell is specified to become the founder cell of the primary root meristem through the MONOPTEROS7-mediated movement of TMO7 from the embryo into the upper cell of the extraembryonic suspensor (Schlereth et al. 2010). Considering the fact that embryos defective in this process lack a root entirely, it would be interesting to see whether the factors affecting TMO7 movement would result in rootless seedlings (Bennett and Scheres 2010). Given the fact that plant development is continuous over a plant's lifetime, intercellular signaling appear to be inevitable and critical throughout its physiology and morphogenesis, marking a hint that temporal expression and activity of mobile protein entities can be numerous. Though our understanding so far extensively advanced only for those who have been evidently shown to be moved either actively or passively by impacting PD SEL, the plentitude of evidences for other proteins that can act non-cell autonomous are continuously reported. Lee et al. (2006) and Rim et al. (2011) independently reported that around 20 % of transcription factors examined in their large-scale screen of NCA-TFs showed the capacity of movement, despite not aligned to follow either active or passive transport.

7.3 Macromolecular Transport in Phloem

In addition to these short range movement of TFs, plant development is also achieved by a global plant communication network in which macromolecules can also act as long-distance trafficking signals (Narvaez-Vasquez et al. 1995; Oparka and Cruz 2000). To establish this network, plants have evolved themselves with a

functional domain of specialized PD interconnecting sieve elements (SE) and companion cells (CC) (Schulz 1998; Crawford and Zambryski 1999). From a large body of evidences accumulated over past years, one can assume that PD is essential for phloem function because macromolecules synthesized in CCs are transported to enucleate SEs (Imlau et al. 1999; Stadler et al. 2005). Also, the studies which aimed to figure out such macromolecular signal(s) which get sophisticated by this supramolecular express highway to reach their destination have made a well-documented history of experimental approaches demonstrating an unambiguous long-distance regulation of plant development. This history is majorly for proteins, peptides, small RNA, and mRNA, all of which were shown to convey information in a non-cell-autonomous or systemic fashion (Ruiz-Medrano et al. 2001; Ding et al. 2003; Lough and Lucas 2006; Kehr and Buhtz 2008). For an example, the long-distance signaling in flowering time control is initiated by the photoperiod sensing organ (leaf) where moving florigen is expressed, but its phenotypic outcome is in spatially separated site of response (shoot apical meristem, SAM) (Dennis et al. 1996). As a major breakthrough in phloem-mediated cell signaling, analyses of phloem sap (exudates) of various plants have incredibly thrown many surprises. These include not only the presence of approximately a thousand soluble proteins but also their dynamic nature, distinctness from the total plant protein profiles and the discovery that these phloem proteins move source-to-sink direction and shows a wide range of functional diversity such as proteins with RNA-binding functions, signal transduction/plant defense, protein synthesis, cell cycle, cell fate, and metabolism (Fisher et al. 1992; Schobert et al. 1998; Hayashi et al. 2000; Hoffmann-Benning et al. 2002; Kuhn et al. 1997; Ham et al. 2009; Lin et al. 2009). Most importantly, a specialized capacity of these proteins to increase the PD SEL was shown to mediate their own cell-to-cell transport into SEs, suggesting a similar mode of mechanisms that underlie the systemic translocation of endogenous phloem macromolecules (Balachandran et al. 1997; Ishiwatari et al. 1998; Rojas et al. 2001; Stadler et al. 2005).

7.3.1 Florigen, a Holy Grail in Plant Biology

Day length-dependent phloem-based signaling was first demonstrated in spinach flowering. The results of this study have concluded that the substance or stimulus produced in leaves in response to day length is transported to the growing point (Knott 1934). Later subsequent studies individually supported this observation by extending to other species such as *Perilla* and *Sinapis alba* and were able to coin the term "florigen" (flower former) for the photoperiodic stimulus, which is perceived in the leaves and transmitted to the shoot apex (Chailakhyan 1936; King and Zeevaart 1973; Bernier et al. 1993). Though several physiological evidences adorned this transmissible flowering stimulus concept, it took 70 years to identify such stimulus mainly because a new perspective in search of florigen was possible by the convergence of classical physiological studies and molecular genetic approaches (Zeevaart 2006). In *Arabidopsis,* CONSTANS (CO), a nuclear

zinc-finger protein, and FLOWERING LOCUS T (FT), a RAF-kinase-inhibitor-like protein, both of which are specific to leaf phloem companion cells and are not expressed in the shoot apex, are the main components of the long-day (LD)-dependent pathway and were shown to mediate the effect of day length on flowering (Kardailsky et al. 1999; Kobayashi et al. 1999; Putterill et al. 1995). Normally in LD conditions, expression of FT is induced by the upregulation of CO and its protein stabilization (Samach et al. 2000). Using tissue-specific and constitutive promoters, a signaling role for CO was demonstrated that ectopic expression and companion cell-specific expression of CO cause early flowering, but not when expressed from a shoot meristem-specific promoter in the *co* mutant background (An et al. 2004). The notion that CO itself can act as a florigen was discounted as companion cell-specific expression of a CO-green fluorescent protein (GFP) fusion protein did not show a capacity to traffic beyond the cells in which it was transcribed (An et al. 2004; Ayre and Turgeon 2004). However, the CO-GFP fusion protein was still able to rescue flowering in the *co* mutant background. Subsequently, a same set of experiments were conducted to test a role for FT and/or FT mRNA as the phloem-mobile florigenic signaling agent(s) using a range of tissue-specific promoters. In contrast to CO expression, when FT was overexpressed in the shoot apex, it could alone induce early flowering, as does expression of CO from companion-cell-specific promoters. These studies, together with the grafting experiments in *Arabidopsis,* speculated that FT protein might be the mobile signal or, that FT controls the synthesis of a mobile, small substance to induce flowering (An et al. 2004; Ayre and Turgeon 2004). There is a possibility that FT mRNA could also act as a signaling molecule since the core of phloem sap mRNAs and RNA-binding proteins (RBPs) can influence events taking place in the meristem (Xoconostle-Cazares et al. 1999; Yoo et al. 2004; Kim et al. 2001; Haywood et al. 2005). This possibility was tested by conducting a series of heterografting studies. In an elegant experiment, when Lifschitz et al. (2006) grafted the stock tomato plant overexpressing SINGLE FLOWER TRUSS (SFT), a tomato FT homolog with the *sft* mutant scion, the floral stimulus could cross the graft union, but SFT mRNA was not detected in the flowering receptor shoots, not supporting a role for the long-distance trafficking of FT mRNA in floral induction. The results were consistent with that of other recent studies conducted on *Arabidopsis* and rice using GFP fusions of FT and Hd3a, the rice ortholog, under the control of vascular tissue-specific promoter. Here, it is evident that FT and Hd3a could move in the phloem to the shoot apical meristem (SAM) to induce flowering, but both mRNAs were not detectable in shoot apex, disproving a definitive role for FT mRNA in long-distance signaling (Corbesier et al. 2007; Jaeger and Wigge 2007; Lin et al. 2009; Mathieu et al. 2007; Tamaki et al. 2007). Though the evidences presented so far with respect to FT and/or FT mRNA to be phloem mobile appear to be different between various species and photoperiodic response types, a commonality exists in all that either FT or a product of FT is the flower-inducing signal. A 20-kDa FT might diffuse readily into the phloem stream as a result of the unusual nature of companion cell PD having the SEL around 67 kDa (Stadler et al. 2005). But in case of *Cucurbita moschata* plants, one could assume that a tight

control over the exit of FT is possible from the companion cells (Lin et al. 2009). Recently, an endoplasmic reticulum (ER) membrane protein, FT-INTERACTING PROTEIN 1 (FTIP1), was reported as an essential regulator required for FT protein transport (Liu et al. 2012). The *ftip*1 mutant exhibits late flowering under long days and the compromised FT movement to the shoot apex. The interaction between FTIP1 and FT in phloem companion cells is required for FT export from companion cells to sieve elements. These results provide a mechanistic understanding of florigen transport demonstrating that FT moves in a selective manner. Further studies at the molecular and cellular levels can evidently prove the detailed mechanisms involved in FT protein movement and its targeted release from the protophloem and intercellular movement in the apex.

7.3.2 CmPP1 and CmPP2

The role of PD in phloem and plant function was supported by a direct experimental evidence that CmPP1 and CmPP2, the proteins present within the phloem sap of *Cucurbita maxima,* have the capacity to interact with PD to (1) induce a significant increase in SEL (20–40 kDa) and (2) mediate their own cell-to-cell transport (Balachandran et al. 1997). Based on microinjection experiments the authors could well be able to show that phloem proteins ranging from 10 to 200 kDa induced an increase in SEL to the same extent, suggesting that protein unfolding appears to be essential in plasmodesmal trafficking (Balachandran et al. 1997). Thus one could convincingly admit the insight that the ways in which higher plants orchestrate events at the whole-plant level may be regulated by the capacity of PD to allow selective trafficking of macromolecules, though such regulatory mechanisms are yet to be uncovered at molecular and cellular level.

7.3.3 CmPP16 and CmPP36

CmPP16, *Cucurbita maxima* phloem protein 16, found in phloem sap, is an important NCAP to have been shown not only to move itself but also to possess the characteristics that are likely required to mediate mRNA delivery into the phloem translocation stream and thereby advancing the emerging picture of non-cell-autonomous regulation of gene expression in plants (Xoconostle-Cazares et al. 1999). A substantial finding added more flavor to the aforementioned picture because the movement of CmPP16 appeared to be regulated by the interaction with a potential PD receptor CmNCAPP1 (*C. maxima* NON-CELL-AUTONOMOUS PATHWAY PROTEIN1). In an another attempt, CmPP36, a pumpkin phloem 36-kDa protein belonging to the cytochrome b5 reductase family, becomes competent to follow regulated cell-to-cell transport when its N-terminal membrane targeting domain is cleaved to follow

PD-phloem transport pathway, though the potential PD targets of CmPP36 are yet to be reported (Xoconostle-Cazares et al. 2000).

7.3.4 CmHSC70

By showing the subclasses of *C. maxima* heat shock cognate 70 (CmHSC70-1 and CmHSC70-2) chaperones to follow the PD non-cell-autonomous translocation pathway using their SVR (short variable region) motif, the cloud surrounding the sky of roles played by the reported chaperone machinery in regulating both protein and RNA entry, long-distance movement, and exit from phloem seems to have been cleared, despite their target cargos are yet to be elucidated (Aoki et al. 2002). It is conceivable from these independent studies that the way by which higher plants orchestrate events at the whole-plant level follows the PD-connected functional ST-CC complex-mediated long-distance translocation pathway in a regulated manner and that a conserved mechanism which facilitates transport of protein-RNA complex is a similar mechanism like viral MPs to facilitate the entry of nucleic acids into SEs.

7.3.5 CmRBP50

In plants, recently, the establishment of non-cell autonomy of some ribonucleoprotein (RNP) complexes and of the presence of a unique population of mRNA species within the phloem translocation stream and their long-distance trafficking have been regarded to influence a range of processes, including organ development, systemic gene silencing, and pathogen defense (Xoconostle-Cazares et al. 1999; Lough and Lucas 2006; Baumberger et al. 2007; Kehr and Buhtz 2008). Some of these phloem-mobile mRNAs are thought to move as RNP complexes. A study, which aimed to decipher the nature of the proteins and mRNA species that constitute phloem-mobile RNP complexes, identified and characterized a 50-kDa pumpkin (*Cucurbita maxima* cv Big Max) phloem RNA-binding protein (RBP50) that is evolutionarily related to animal polypyrimidine tract binding proteins. The findings from this combinatorial study involving *in situ* hybridization, immunolocalization, heterografting, and several biochemical analyses collectively confirmed that RBP50 functions as a non-cell-autonomous RBP and traffics as RNP complex by binding with polypyrimidine tract motif of mobile mRNA species (Ham et al. 2009). Later, it was shown that such binding is facilitated by the core assembly of six additional phloem proteins with CmRBP50, which in turn requires phosphorylation at the C-terminus to make such a complex, thus providing an enhanced binding affinity for phloem-mobile mRNAs carrying polypyrimidine tract-binding motifs (Li et al. 2011). Though the authors correlated this property with the effective long-distance translocation of bound mRNA to the target tissues, the physiological outcome concerning this property is still lacking.

7.3.6 *Phloem RNAs: Role in Systemic Regulation of Plant Development*

The issues that in recent past came under the spotlight are greatly exciting as they suggest the possibility that phloem-mobile RNA species regulate plant development. The notion that the macromolecules present in the sieve tube, including mRNA molecules, are in the process of long-distance trafficking is supported mainly by experiments in which species-specific mRNA molecules pass through the unions of heterografts. In one study, heterografting experiments in potato showed that full-length mRNA of the BEL1-like transcription factor (StBEL5) moves from leaves to stolon tips through the phloem, suggesting that StBEL5 acts as a long-distance signal molecule (Banerjee et al. 2006). In an another study, heterografting in melon identified only 6 of 43 randomly selected transcripts in the scions, suggesting systemic trafficking ability is not for all phloem sap mRNA molecules (Omid et al. 2007). The findings that phloem sap contains a large range of small RNAs, including small interfering RNAs (siRNAs) directed against transposons, transgenes, or viruses in the case of viral infection, and the siRNA are non-cell autonomous (Kehr and Buhtz 2008) are the strengthening bricks of the wall of the speculation that RNAs can be long-distance signals. More exciting is the detection of 3 miRNAs (miR156, miR159, miR167) in pumpkin phloem sap and the subsequent detection of almost 32 well-known plant miRNAs belonging to 18 different families identified in phloem sap of *Brassica napus* (Sauer 2007). Yoo et al. (2004) have gone one step further with microinjection assays by showing that the trafficking of small RNAs is regulated through PD and by identifying a small RNA-binding protein required to transport such miRNAs cell to cell. However, the phenomenon that long-distance signaling is mediated through phloem-mobile RNAs is still under the debate mainly due to the lack of direct proof and awaits the followings discoveries: how many transcripts identified so far are phloem mobile, and most importantly, what exactly the biological roles are for most transcripts in enucleated SEs or their target sink tissues? Overall, from these independent studies on macromolecule transport in phloem, it is conceivable that higher plants orchestrate cellular processes at the whole-plant level through the highly regulated long-distance translocation pathways and that a conserved mechanism involving phloem proteins which facilitate trafficking of RNA molecules is a similar mechanism to that employed by viral MPs to facilitate the entry of nucleic acids into SEs.

7.4 Regulatory Mechanisms for Intercellular Protein Movement

Previous studies on intercellular movement of macromolecules in plants came up with two possible modes through PD: selective and nonselective movement (Crawford and Zambryski 2000, 2001). Proteins that fall under the former group

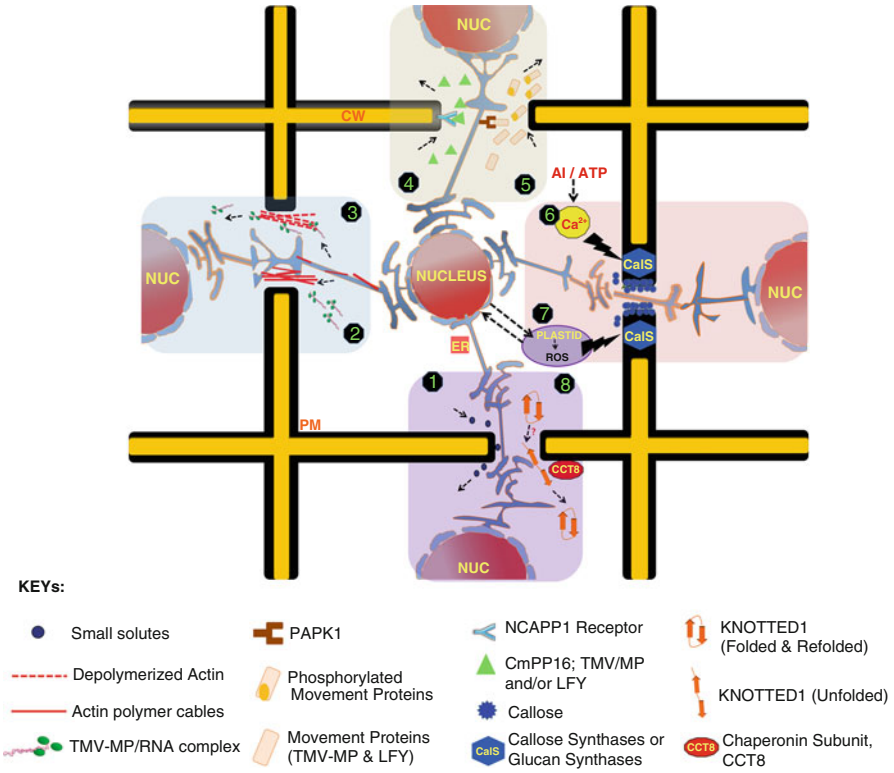

Fig. 7.6 Schematic summary of major regulatory mechanisms of PD transport of non-cell-autonomous cellular viral and proteins by establishing symplasmic domains between cells. (1) A mechanism involving simple diffusion of small molecules such as nutrients and ions is shown. (2) and (3) Schematic illustration of cytoskeleton-mediated intercellular movement of proteins is shown. Plasmodesmal-located actin polymers [the polymerized actin is represented in thick red line (2)] may restrict the movement and spread of TMV-MP/RNA complex. Interaction of this complex with cytoskeletal elements may depolymerize actin complex by an unknown mechanism [see depolymerized state of actin as shown as *dashed line* (3)], leading to elevated PD permeability, which would provide easy access to the transport channel, thus facilitating spread of large TMV-MP/vRNA complex from cell-to-cell. (4) and (5) PD transport of some of the transcription factors and viral movement proteins occurs by gating of PD-localized receptors. PD nanochannels are dilated by the binding of a "gate open" (protein kinase and glycosylating enzymes) to its cognate PD-gating NCAPP1 receptor and phosphorylating both receptors and molecules such as CmPP16 and/or TMV-MP that are free to move within the cytoplasm. Then the size of these molecules will permit passage through the dilated nanochannels and they can diffuse into neighboring cells (4). (5) A role of a PD-associated protein kinase (PAPK) in regulating the function of TMV-MP/other NCAPs in cell-to-cell trafficking is brought about by the posttranslational modification of such moving proteins. (6) and (7) PD transport of proteins is restricted by callose sphincters, which can be regulated by Ca^{2+} ions and/or ROS. In response to aluminum (Al) metal ion stress and/or imbalance in physiological ATP level, increase in physiological endogenous Ca^{2+} ions negatively modulates PD SEL by upregulating callose-synthesizing enzymes (CalS) through an as yet unknown pathway that leads to overaccumulation of PD callose to restrict the movement of molecules, suggesting that Ca^{2+} is an important factor for PD regulation. In other mechanism, ROS-mediated interorganelle cross-talk regulates the expression of CalS to gate PD. As an example,

actively make the PD aperture larger (size exclusion limit, SEL) to allow their trafficking into the neighboring cell. Proteins that belong to the latter group show simple passive diffusion through PD. LFY is an elegant example to this type and its movement was shown to be nonselective using GFP fusion proteins controlled by the L1-specific promoter (Wu et al. 2003). It is also consistent with the fact that LFY does not seem to have any trafficking signal sequence. It provokes an insight that proteins can diffuse cell to cell by default if their size is smaller than the PD SEL. Formation of endogenous protein complexes or subcellular sequestration also prohibits their intercellular movement. Selective NCAPs need to interact with component(s) associated to the selective non-cell-autonomous trafficking pathway. The HD of KN1 is necessary and sufficient for intercellular trafficking of the KN1 protein or fused cell-autonomous protein (Kim et al. 2005). Meanwhile, mobility of CPC and SHR is achieved by multiple regions within the respective proteins (Gallagher et al. 2004; Kurata et al. 2005). Protein mobility can also be knotted with the subcellular localization. A mutation in nuclear localization signal located at the N-terminus of KN1 HD abolished the ability of intercellular trafficking. In case of SHR, both cytoplasmic and nuclear localization are necessary for trafficking (Gallagher et al. 2004; Gallagher and Benfey 2009). A mutation in CPC prevented movement and caused an increase in cytoplasmic localization, suggesting an overlapping mechanism between movement and subcellular localization (Kurata et al. 2005). In addition to these intrinsic features of NCAPs, there are a number of extrinsic features that have been shown to influence macromolecular transport (see Table 7.2; Fig. 7.6) though majority of them have to be yet elusive. A notion that specific PD receptor proteins recognize and regulate the structurally distinct NCAPs has been demonstrated by the identification of a non-cell autonomous protein pathway receptor, NCAPP1. Though they were initially reported to specifically traffic CmPP16 and TMV-MP, the developmental abnormalities caused by their dominant negative version extended their selectivity over a range of NCAPs, specifically for LFY as this phenotype resembled LFY overexpression phenotype (Lee et al. 2003). Callose, a β-1,3-glucan, is the best-studied regulatory component of PD, and its deposition around neck constricts the channel and limits macromolecular traffic (Zavaliev et al. 2011). The consequence of the loss-of-function mutations in AtGSL8, with abnormal stomatal patterning, is the result of movement of the

Fig. 7.6 (continued) nucleus-plastid cross-talk is shown. In this, shifts in the redox states of plastids (*purple*) in response to ROS signal the nucleus (Nuc) to differentially regulate CalS and so accumulate callose in and around PD to restrict intercellular transport. (8) Mechanism involving a selective trafficking of KN1 and its homologs is shown. CCT8, the chaperonin complex, is required in destination cells for successful KN1 trafficking through refolding translocated, partially unfolded (indicated by shape change) by as-yet unknown mechanism to allow transport through the PD. Co-diffusion of small molecules is also possible through this dilated PD (not shown). *CW* cell wall, *Nuc* nucleus, *PM* plasma membrane, *ER* endoplasmic reticulum, *Al/ATP* aluminum or adenosine triphosphate, *CalS* callose-synthesizing enzymes, *ROS* reactive oxygen species

normally cell-autonomous protein SPEECHLESS (SPCH), implying a role for callose level in protein movement (Guseman et al. 2010). A mutant, *gfp-arrested trafficking 1* (*gat1*), was isolated with reduced GFP unloading from the phloem and diffusion into the root meristem. GAT1 encodes an m-type thioredoxin; the *gat1* mutation results in an accumulation of reactive oxygen species (ROS) and consequently increases callose at PD. *gat1* mutations are seedling lethal with defects in meristem formation presumably due to decreased intercellular protein movement (Benitez-Alfonso et al. 2009). Considering the relation between callose deposition and ROS, there will be a range of mobile proteins that potentially control themselves their transport by regulating callose levels directly or indirectly. By maintaining ROS gradients through differential expression of peroxidases in the cells of vascular tissues as well as in the lateral root cap (LRC) and cells in the transition and elongation zones, a mobile bHLH family of TF, UPBEAT1 (UPB1), controls *Arabidopsis* root development (Tsukagoshi et al. 2010). Another mobile h-type thioredoxin (Trx-h9) has also been identified to be required for normal plant development by regulating ROS levels (Meng et al. 2010). It was recently shown that *lfy* mutants increased callose accumulation and pronounced resistance to pathogens, suggesting that LFY inhibits callose formation (Winter et al. 2011). All these three proteins might also facilitate other proteins to get access to PD channel during their transport mechanism since they modulate ROS and/or callose levels directly. By contrast, PD-mediated transport was increased in *increased size exclusion limit 1* (*ISE1*)-silenced tissues and *ise1* mutants, although both plants produced increased amounts of ROS compared with their wild-type plants (Stonebloom et al. 2009). These different effects of ROS on PD-mediated transport might be due to the accumulation of ROS from different subcellular compartments. In fact, elevated level of ROS in *ISE1*-silenced plants is due to an increase in ROS production in mitochondria, while loss of GAT1 function likely results in a local oxidative shift in plastid (Stonebloom et al. 2012), indicating the differential effect of mitochondrial and plastidal redox states on intercellular transport through PD. Implications into the intracellular trafficking of mobile proteins would also be informative for the intercellular trafficking. Endosomal-mediated movement of proteins through PD was demonstrated recently for TMV-MP and CaLCuV (cabbage leaf curl virus)-MP. These proteins interact with *Arabidopsis* synaptotagmin, SYTA. In *syta* knockdown lines, movement of CaLCuV-MP and TMV-MP is delayed or inhibited (Lewis and Lazarowitz 2010). Similarly, it is noted that in animals the endosome/recycling endosome regulates transport of cargo proteins between cells through a process of unconventional secretion (Joliot et al. 1998).

7.5 Perspectives

Intercellular trafficking of proteins and their RNAs in plants has indeed been effective in a range of developmental signaling that instructs a plant to carry out their day-to-day activities. One of the most provocating aspects of symplasmic signaling

via transport of proteins hides under the answers to the questions that how extensive are the non-cell-autonomous proteins (NCAPs) directed to the adjacent cells through the unique cytoplasmic bridges and what does it make them to become movement competent. Though the last decade has accumulated a handful of reports that "seem" to have aimed to answer the former questions (Fig. 7.6), considering the scenario that a lot of proteins shown to be mobile and to orchestrate plant morphogenesis, cell fate determination, and even defense response, current knowledge on intercellular macromolecular trafficking is still in its infancy. So it is pretty sure that the future will be bright for those who can effectively look for and utilize the diverse genomic, cellular, and genetic breakthroughs. In other words, this search will also get benefits by the wealth of studies that are focused to uncover the plasmodesmal components.

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Chapter 8
Plasmodesmata: New Perspectives on Old Questions

Robyn L. Overall, Danny Y.T. Liu, and Deborah A. Barton

Abstract The progress so far and challenges remaining in developing a functional model of the macromolecular architecture of plasmodesmata are discussed. Details of the macromolecular components identified within plasmodesmata are summarised. Electron tomography and correlative microscopy techniques are explored as potential avenues to overcome the challenges in developing an accurate three-dimensional model of the macromolecular architecture of plasmodesmata. In recent years, some areas of plasmodesmatal biology have been left ignored, largely because the technologies required to advance them have been considered too difficult. For example, there have been no electrophysiological studies of plasmodesmata in the last decade and consequently no advances in our understanding of the rapid regulation of the permeability of plasmodesmata. There has also been no advance on the question of heterogeneity of function of the plasmodesmata within a wall and potential avenues to address this question are considered.

Keywords Correlative microscopy • Cytoskeleton • Electron tomography • Electrophysiology • Endoplasmic reticulum • Macromolecular architecture • Plasmodesmata • Sphincter

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Abbreviations

CMV	*Cucumber mosaic virus*
ER	Endoplasmic reticulum
FA	Functional assay
FM	Fluorescence microscopy
FPF	Fluorescent protein fusion observed using fluorescence microscopy
GFP	Green fluorescent protein
GPI	Glyco phosphatidyl inositol
IFM	Immunofluorescence microscopy
ITEM	Immune-gold transmission electron microscopy
MP^{TMV}	Movement protein of *Tobacco mosaic virus*
MP^{TVCV}	Movement protein of *Turnip vein-clearing mosaic virus*
NCAP	Non-cell autonomous protein
PA	Protein analyses such as immune-blotting and proteomics
PD	Plasmodesmata/plasmodesma
RFP	Red fluorescent protein
ROS	Reactive oxygen species
SEL	Size exclusion limit
TEM	Transmission electron microscopy
YFP	Yellow fluorescent protein

8.1 Introduction

This book journeys from discussion of the very first description of plasmodesmata (PD) in 1880 (see Sect. 1.2) to the latest on the molecules that regulate symplasmic transport (see Chaps. 2 and 3). It highlights the spectacular advances in our understanding of intercellular communication in plants that have been spawned through molecular biology. There is an emerging picture of a web of transcription factors and other proteins that move between cells via plasmodesmata to regulate developmental patterning (see Chap. 7). We have insights into how PD are formed and modified (see Sect. 2.4), and some of their components are beginning to be identified. Despite these advances, there are areas of PD biology that have remained almost completely unexplored. Indeed some questions posed nearly four decades ago in the first book on PD (Gunning and Robards 1976) remain unanswered. This chapter will briefly reflect on some of the progress that has been made in the understanding of PD since 1976, particularly in the identification of macromolecular components of PD, while highlighting other areas in which progress has been limited.

8.2 Macromolecular Architecture of Plasmodesmata

Articulation of an accurate three-dimensional structural model of PD with all of the functional components identified and localised at high resolution is a long way off. While molecular components are being identified, their positions have not been pinpointed to structures within the PD. Indeed, even the details of the structure of PD are yet to be unambiguously resolved.

8.2.1 Macromolecular Constituents of Plasmodesmata

There is growing list of proteins and other macromolecules that are found to be associated with PD, as opposed to the non-cell autonomous macromolecules described in Sect. 1.4.1 and Chap. 7. The identity and pathway to discovery of these plasmodesmata-associated macromolecules are summarised in Table 8.1 and are discussed below in terms of their association with various aspects of cell biology. These proteins and their known subcellular localisations are also summarised in Fig. 8.1.

8.2.1.1 Carbohydrates and Related Proteins

Callose is an important component of PD involved in aperture regulation (see Sects. 2.3 and 3.3.2). It was originally visualised in onion epidermal pit fields by staining with aniline blue (Currier and Strugger 1956) and has been subsequently immunolocalised to PD using TEM (Northcote et al. 1989; Radford and White 1998). Callose is considered a *bona fide* plasmodesmatal marker, being used to co-localise plasmodesmatal proteins by fluorescent staining or immunolocalisation (e.g. Blackman and Overall 1998; Baluška et al. 1999; Blackman et al. 1999; Levy et al. 2007; Thomas et al. 2008; Faulkner et al. 2009; Simpson et al. 2009). Concomitantly, antibodies raised against mung bean membrane fractions with callose synthase activity immuno-decorated cucumber sieve plates and punctate spots along onion epidermal walls (Delmer et al. 1993); however, the 65 kDa polypeptide recognised by this antibody may have an entirely different identity (Delmer and Amor 1995). The Arabidopsis GLUCAN SYNTHASE-LIKE8 (GSL8) protein was isolated from a sensitised mutagenesis screen for aberrant stomatal patterning (Guseman et al. 2010). Consistent with a role at PD, *gsl8* mutants exhibited diminished callose accumulation at PD and greatly increased plasmodesmatal SEL that allowed even triple-fused GFP (81 kDa) to move cell to cell (Guseman et al. 2010). Also, an Arabidopsis β-1,3-glucanase (AtBG_ppap, *Arabidopsis thaliana* β-1,3-glucanase putative plasmodesmata-associated protein) has been isolated from a plasmodesmata-rich cell fraction and cloned (Levy et al. 2007). When fused with GFP and expressed transiently and stably in *N. benthamiana* and *N. tabacum*, respectively, it appeared to localise to punctate spots along the cell walls. Mutants exhibited decreased

Table 8.1 Macromolecules associated with plasmodesmata

Macromolecule	Method of discovery	Evidence for plasmodesmatal association—initial and subsequent	Function	References
α-Amylase	Anti-α-amylase antibodies used to immunoprobe barley aleurone	Anti-α-amylase antibodies labelled PD and the wall region around PD (ITEM)	Hydrolysis of endosperm stores and cell walls	Gubler et al. (1987)
Actin	Anti-actin antibodies used to immunoprobe *Nephrolepis exaltata* rhizome and barley root tips	Anti-actin antibody labelled throughout PD of *N. exaltata* and barley [1] and *Chara corallina* [2] (ITEM); rhodamine-phalloidin labelled filamentous structures crossing cell walls of barley coleoptile epidermis and *Nicotiana plumbaginifolia* suspension culture cells (FM)	Possibly structural and/or regulatory role	[1] White et al. (1994) [2] Blackman and Overall (1998)
Actin-related protein 3 (ARP3)	Anti-*Dictyostelium discoideum* ARP3 antibody used to immunoprobe maize root tips	Anti-ARP3 antibodies labelled cell wall puncta in maize roots (IFM) and throughout PD and pit fields of maize root cells (ITEM)	Unknown, possibly involved in nucleation and/or organisation of filamentous actin	van Gestel et al. (2003)
At-4/1	Movement protein of *Tomato spotted wilt virus* found to interact with At-4/1 through yeast two-hybrid screen	Localised to paired puncta along cell walls (FPF)	Unknown, possibly targeted by virus for cell-to-cell movement	Paape et al. (2006)

ATPases (activity of)	Cytochemical staining for ATPase activity [1]	ATPase-induced lead precipitate at PD of *Nicotiana tabacum* vascular tissue [1], barley root tips [2] and virus-infected corn leaves [3] (FA, TEM), especially around openings of PD ([2] FA, TEM)	Possibly involved in active transport through/ around PD	[1] Cronshaw (1980) [2] Belitser et al. (1982) [3] Chen et al. (1994)
β-1,3-Glucanase	Isolated and identified from PD-enriched Arabidopsis cell fraction by proteomics	Punctate signals in bombarded *N. benthamiana* and transgenic *N. tabacum* cell walls ([1] FPF), co-localisation with aniline blue staining in transgenic *N. tabacum* ([1] FPF), reduced GFP cell-to-cell movement and increased callose accumulation in knockout mutants ([1] FA), detected in PD-rich *Chara* wall fractions ([2] PA)	Likely to regulate cell-to-cell communication by degrading callose at PD	[1] Levy et al. (2007) [2] Faulkner et al. (2005)
Ca^{2+} dependent protein kinase (CDPK)	Maize seedling mesocotyl immune probed with antiserum produced against an Arabidopsis CDPK	Anti-CDPK serum labelled puncta in isolated cell walls (IFM) and detected a single band in proteins extracted from PD-enriched purified cell walls (PA)	Likely to be intrinsic membrane protein, possibly involved in rapid PD regulation	Yahalom et al. (1998)
Callose	Aniline blue staining of onion epidermal cells [1]	Staining of onion epidermal pit fields with aniline blue, maintained after plasmolysis ([1] FM); anti-callose serum labelled PD in bean seedling root tips ([2] ITEM)	Sealing of PD	[1] Currier and Strugger (1956) [2] Northcote et al. (1989)

(continued)

Table 8.1 (continued)

Macromolecule	Method of discovery	Evidence for plasmodesmatal association—initial and subsequent	Function	References
Callose synthase-associated protein	Antibodies against mung bean membrane protein extract with callose synthase activity used to immunoprobe cucumber and onion	Anti-callose synthase antibodies labelled cucumber seedling sieve plates and spots along onion epidermis cell wall ([1] FM). However, the immunoreactive protein appears not to be a callose synthase [2]	Unknown, presumably callose synthesis [1]	[1] Delmer et al. (1993) [2] Delmer and Amor (1995)
Calreticulin	Anti-calreticulin antibody used to immunoprobe maize root tips	Anti-calreticulin antibody labelled puncta along cell walls which co-localised with immunolocalised callose during plasmolysis (IFM), anti-calreticulin antibodies labelled PD (ITEM)	Unknown, possibly involved in gating PD	Baluška et al. (1999)
Casein kinase 1-like 6 (CKL6)	Arabidopsis ortholog of tobacco PAPK having similar substrate preference [1]	Puncta in bombarded tobacco and Arabidopsis cell walls ([1, 2] FPF), co-localisation with MP^{TMV}-GFP marker in bombarded tobacco ([1] FPF), close localisation with MP^{TMV}-GFP in bombarded Arabidopsis ([2] FPF)	Possible regulation of NCAP trafficking	[1] Lee et al. (2005) [2] Ben-Nissan et al. (2010)
Centrin	Onion root tips and cauliflower florets immunoprobed with anti-centrin antibodies	Punctate wall labelling with anti-centrin antibodies and co-localisation with anti-callose antibodies (IFM), anti-centrin antibodies labelling necks of PD (ITEM)	Possibly modulates PD aperture in neck region	Blackman et al. (1999)

Connexin-like	Antibodies against rat liver connexin and putative soybean connexin used to immunoprobe cells of soybean root culture [1]	Anti-connexin antibodies label puncta at cell-to-cell contact points of cultured soybean cells ([1] IFM), PD of maize mesocotyl ([2] ITEM) and puncta at protoplast surfaces of *Helianthus annuus* and *Vicia faba* ([3] IFM). However, the immunoreactive protein is possibly not connexin [4]	Unknown, possibly structural	[1] Meiners and Schindler (1989) [2] Yahalom et al. (1991) [3] Schulz et al. (1992) [4] Mushegian and Koonin (1993)
CRINKLY4 (CR4)	Maize endosperm immunoprobed with anti-CR4 antibodies	Anti-CR4 antibodies labelled throughout PD (ITEM)	Signalling pathway for aleurone layer development	Tian et al. (2007)
Dehydrin-like	*Cornus sericea* stem tips immunoprobed with immune serum against 24 kDa dehydrin-like protein	Immune serum labelled PD, predominantly around collar region (ITEM)	Possible role in protecting PD from desiccation/freezing-induced injury	Karlson et al. (2003)
DEVH box RNA helicase (ISE2)	Mutagenised Arabidopsis embryos exhibited increased intercellular flow of tracer dyes [1]	PD in mutant embryos maintained high SEL ([1, 2] FA), mutant embryos possessed more branched PD ([2] TEM)	Unknown, possibly involved RNA silencing and in PD formation	[1] Kim et al. (2002) [2] Kobayashi et al. (2007)
DEAD box RNA helicase (ISE1)	Mutagenised Arabidopsis embryos exhibited increased intercellular flow of tracer dyes [1]	PD in mutant embryos maintained high SEL ([1, 2] FA), mutant embryos possessed more branched and twinned PD ([2] TEM), silencing of ISE1 caused increased cell-to-cell trafficking via PD in mature *N. benthamiana* leaves ([2] FA)	Unknown, possibly mediates mRNA processing in mitochondria	[1] Kim et al. (2002) [2] Stonebloom et al. (2009)

(continued)

Table 8.1 (continued)

Macromolecule	Method of discovery	Evidence for plasmodesmatal association—initial and subsequent	Function	References
Flowering Locus T (FT)-Interacting Protein1 (FTIP1)	Yeast two-hybrid screen for proteins interacting with FT	Expression in the ER membrane and at puncta along cell walls of agroinfiltrated *N. benthamiana* (FPF), the puncta co-localised with aniline blue staining	Role in transporting FT between companion cells and sieve elements, broader role in control of flowering	Liu et al. (2012)
Glucan synthase-like 8 (GSL8)	Forward genetics, mutagenesis of sensitised stomatal patterning Arabidopsis mutant	GSL8 mutant leaf epidermis exhibits almost no aniline blue staining and increased PD size exclusion limit (FA)	Callose synthesis to regulate intercellular trafficking of cell fate determinants	Guseman et al. (2010)
Heat shock cognate 70 (HSC70)-like proteins	Anti-wheat heat shock protein 70 (HSP70) antibodies used in immunoblots against PD-enriched cell fractions and pumpkin phloem sap [1]	Anti-HSP70 antibodies labelled 70 kDa band in PD-enriched cell fractions and phloem sap ([1] PA); C-terminal motif enables gating of and movement through PD ([1] FA); detected in PD-rich *Chara* soluble fractions ([2] PA)	Possibly binds and folds macromolecules that pass through PD	[1] Aoki et al. (2002) [2] Faulkner et al. (2005)
Hypothetical protein containing a DUF1218 domain	Proteomic search for Arabidopsis PD proteins	Punctate signals in cell walls of stably transformed Arabidopsis leaves that co-localise with aniline blue (FPF)	Unknown	Fernandez-Calvino et al. (2011)
Microtubule-associated protein 65 (MAP65-5)	Screen of putative Arabidopsis cytokinesis-related genes by translational fusion to GFP	Appeared to cross newly formed cell wall in tobacco BY2 cells (FPF)	Unknown, microtubule binding	van Damme et al. (2004)
Monodehydroascorbate reductase	*N. benthamiana* root cDNA digested and fused to GFP, expressed in *N. benthamiana* leaves using a viral vector and screened for PD localisation	Partial cDNA-GFP fusion localised to spots along cell periphery (FPF), anti-GFP antibodies labelled PD in expressing tissues (ITEM)	Various and unknown	Medina-Escobar et al. (2003)

Monosaccharide transporter	Immunoblotting with antisera against a putative grape hexose transporter, VvHT1, identified a 52 kDa plasma membrane-associated protein [1]	Anti-VvHT1 localisation to PD in cell walls between companion cells and sieve elements as well as parenchyma cells in apple fruit during mid-stage development ([2] ITEM)	Unknown, possible role in trafficking sugars	[1] Zhang et al. (2004) [2] Zhang et al. (2005)
Myosin	Anti-mung bean myosin antibodies [1] or anti-rabbit muscle myosin antibodies [2] used to immunoprobe *Chara corallina* nodal walls [1] or onion, maize and barley roots [2]	Anti-myosin antibodies labelled throughout PD ([1, 2] ITEM), anti-myosin antibodies labelled strands that crossed cell walls at pit fields ([2] IFM)	Unknown, possibly structural or may regulate PD aperture	[1] Blackman and Overall (1998) [2] Radford and White (1998)
Class VIII myosin: Arabidopsis thaliana myosin I (ATM1)	Anti-ATM1 antibody used to immunoprobe Arabidopsis, *Lepidium sativum* and maize root tissue	Anti-ATM1 antibody labelled puncta in cell walls of root tissue ([1, 2] IFM) and wall areas with high PD density ([1] ITEM)	Possibly involved in formation of young cell wall	[1] Reichelt et al. (1999) [2] Baluška et al. (2001)
NADH dehydrogenase subunit	*N. benthamiana* root cDNA digested and fused to GFP, expressed in *N. benthamiana* leaves using a viral vector and screened for PD localisation	Partial cDNA-GFP fusion localised to spots along cell periphery (FPF), anti-GFP antibodies labelled PD in expressing tissues (ITEM)	Various and unknown	Medina-Escobar et al. (2003)
Networked 1A (NET1A)	Screen of an Arabidopsis cDNA GFP fusion library for actin-binding proteins	Puncta in root cell walls in stably transformed Arabidopsis plants under endogenous promoter; these co-localised with aniline blue (FPF); anti-NET1A labels PD in Arabidopsis roots (ITEM)	Possible association with the plasma membrane and actin filaments in PD	Deeks et al. (2012)

(continued)

Table 8.1 (continued)

Macromolecule	Method of discovery	Evidence for plasmodesmatal association—initial and subsequent	Function	References
P45	Antibody against 45 kDa protein from *Chara corallina* nodal wall fraction used to immunoprobe *C. corallina* and onion root tip walls	Anti-P45 antibody labelled mainly the interior of *C. corallina* nodal and onion root tip PD (ITEM), anti-P45 antibody labelling co-localised with aniline blue in *C. corallina* walls (IFM)	Unknown	Blackman et al. (1998)
Pathogenesis response (PRms) protein	Fungus-infected maize radicle immunoprobed with anti-PRms antibody	Anti-PRms antibodies labelled throughout PD and adjoining ER (ITEM)	Possible role in controlling viral infection	Murillo et al. (1997)
PD-associated protein kinase (PAPK)	Kinase activity on NCAPs observed in PD-enriched tobacco cell wall protein fractions	*In vitro* phosphorylation of MP^{TMV} and other NCAPs (FA); isolation of Arabidopsis ortholog (see CKL6)	Possible regulation of NCAP trafficking	Lee et al. (2005)
PD-associated receptor-like kinases (PDRLK)	PD localisation assessed for selected rice RLKs tagged with GFP, medium-throughput [1], proteomic search for Arabidopsis PD proteins [2]	Punctate signals and co-localisation with MP^{TVCV}-RFP in cell walls of agroinfiltrated *N. benthamiana* (FPF) [1], puncta along cell walls in stably transformed Arabidopsis leaves that co-localised with aniline blue stained callose at PD (FPF)	Possibly involved in signal transduction from apoplast	[1] Jo et al. (2011) [2] Fernandez-Calvino et al. (2011)
PD callose-binding protein 1 (PDCB1)	Identified from Arabidopsis suspension cell wall fraction [1], fluorescent tagged PDCB1 localised to punctate structures along cell wall [2]	Puncta along cell walls which co-localised with aniline blue in Arabidopsis ([2] FPF), anti-PDCB1 antibodies labelled fluorescently tagged PDCB1 at necks of PD (ITEM); Arabidopsis overexpression mutants had decreased cell-to-cell movement via PD ([2] FA)	Unknown, binds callose, possibly involved in modulating cell-to-cell trafficking	[1] Bayer et al. (2006) [2] Simpson et al. (2009)

PD germin-like proteins 1 and 2 (PDGLP1; PDGLP2)	Co-immunoprecipitation of PD-enriched tobacco cell wall protein fractions with the NCAP, pumpkin phloem protein16, as bait	Punctate signals and co-localisation with MP^{CMV}-GFP in cell walls of agroinfiltrated *N. benthamiana* (FPF), anti-GFP labelled PD in roots of stably transformed Arabidopsis plants expressing PDGLP1-GFP (ITEM), overexpression of PDGLP1 and PDGLP2 increased PD permeability in roots (FA)	Role in regulating root development	Ham et al. (2012)
PD-located protein 1a (PDLP1a)	Identified from Arabidopsis suspension cell wall fraction [1], fluorescently tagged PDLP1a localised to punctate structures along cell wall [2]	Puncta along cell walls which co-localised with aniline blue and MP^{TMV}-RFP in Arabidopsis ([2] FPF); Arabidopsis expression mutants had affected cell-to-cell movement via PD ([2] FA)	Unknown	[1] Bayer et al. (2006) [2] Thomas et al. (2008)
Pectin	Anti-pectin antibodies used to immunoprobe tomato pericarp [1, 3] and apple pericarp [2]	Anti-pectin antibodies label pit fields ([1, 3] IFM) and area around and within PD ([2, 3] ITEM)	Possibly modulates degree of cell separation and autolysis and/or mechanical properties of cell wall around PD/pit fields	[1] Casero and Knox (1995) [2] Roy et al. (1997) [3] Orfila and Knox (2000)
Pectin methylesterase (PME)	*Linum usitatissimum* seedling hypocotyls immunoprobed with anti-PME antibody	Anti-PME antibody observed throughout PD (ITEM)	De-esterification of cell wall pectins	Morvan et al. (1998)
Peroxidase	Oxidation of 3,3′-diaminobenzidine (DAB) in extracted apple cell wall material [1], $CeCl_3$ treatment or antibodies against class III peroxidases in tomato meristems [2]	Precipitate of DAB and $OsO4_4$ at PD (FA, TEM) [1], precipitate of cerium perhydroxides at PD [2], antibodies to class III peroxidases labelled PD [2]	May effect cell wall cross-linking or cell wall loosening during development of PD	[1] Ingham et al. (1998) [2] Ehlers and van Bel (2010)

(continued)

Table 8.1 (continued)

Macromolecule	Method of discovery	Evidence for plasmodesmatal association—initial and subsequent	Function	References
Putrescine *N*-methyltransferase	*N. benthamiana* root cDNA digested and fused to GFP, expressed in *N. benthamiana* leaves using a viral vector and screened for PD localisation	Partial cDNA-GFP fusion localised to spots along cell periphery (FPF), anti-GFP antibodies labelled PD in expressing tissues (ITEM)	Various and unknown	Medina-Escobar et al. (2003)
Ras-related protein Rab11b	*N. benthamiana* root cDNA digested and fused to GFP, expressed in *N. benthamiana* leaves using a viral vector, and screened for PD localisation	Partial cDNA-GFP fusion localised to spots along cell periphery (FPF), anti-GFP antibodies labelled PD in expressing tissues (ITEM)	Various and unknown	Medina-Escobar et al. (2003)
Remorin	Potato remorin fused to fluorescent protein localised to punctate structures along cell wall	Remorin localised to puncta along cell walls and co -localised with PDLP1 in tobacco (FPF), anti-remorin antibodies labelled PD in tobacco and tomato (ITEM)	Unknown, possibly negative regulator of virus movement	Raffaele et al. (2009)
Class 1 reversibly glycosylated polypeptide (^{C1}RGP) originally identified as a salt-extractable 41 kDa wall-associated protein (SE-WAP41)	Antiserum against 41 kDa protein (SE-WAP41) from maize cell wall protein extract localised to PD [1]	Antiserum (slightly cross-reactive) against SE-WAP41 labelled throughout PD ([1] ITEM), SE-WAP41 identified as belonging to ^{C1}RGP family and Arabidopsis orthologs observed as cell wall puncta which co-localised with aniline blue in transgenic tobacco ([2] FPF), ortholog present in PD-rich *Chara* soluble fractions ([3] PA), overexpression of RGP1 and RGP2 decreased PD permeability ([4, 5] FA)	Possibly involved in sugar transport around PD	[1] Epel et al. (1996) [2] Sagi et al. (2005) [3] Faulkner et al. (2005) [4] Zavaliev et al. (2010) [5] Burch-Smith et al. (2012)

Tetraspanin 3	Proteomic search for Arabidopsis PD proteins	Puncta in cell walls of stably transformed Arabidopsis leaves that co-localise with aniline blue (FPF)	Possibly forms specialised plasma membrane domains at PD	Fernandez-Calvino et al. (2011)
Thioredoxin m3 (GAT1)	Mutagenised Arabidopsis screened for restricted movement of GFP out of root phloem	GFP transport out of root phloem was restricted in mutant Arabidopsis (FA); PD in mutants exhibited altered structure (TEM); mutants accumulated higher levels of callose (FA, FM); ectopic overexpression induced increased cell-to-cell transport of GFP in mature tissues (FA)	Unknown, involved in redox regulation, may maintain symplamic connectivity in meristematic tissues	Benitez-Alfonso et al. (2009)
Tropomyosin-like	Identified from PD-rich *C. corallina* wall fractions by proteomics	Anti-animal tropomyosin antibodies labelled strands across *Chara* walls and puncta in walls of Arabidopsis root tips and leek epidermis, which co-localised with anti-callose antibody labelling (IFM)	Unknown, possibly functions with actin cytoskeleton in PD	Faulkner et al. (2009)
Ubiquitin	*Solanum nigrum* protoplasts immunoprobed with anti-ubiquitin antibody	Anti-ubiquitin antibodies labelled PD (excluding neck regions), especially those PD destined for degradation (ITEM)	Possibly involved in targeting PD and/or PD proteins for degradation	Ehlers et al. (1996)

Macromolecules are listed in alphabetical order, along with the background and method of their discovery, the primary evidence for their localisation and/or association with plasmodesmata, and their proposed functions (if known)

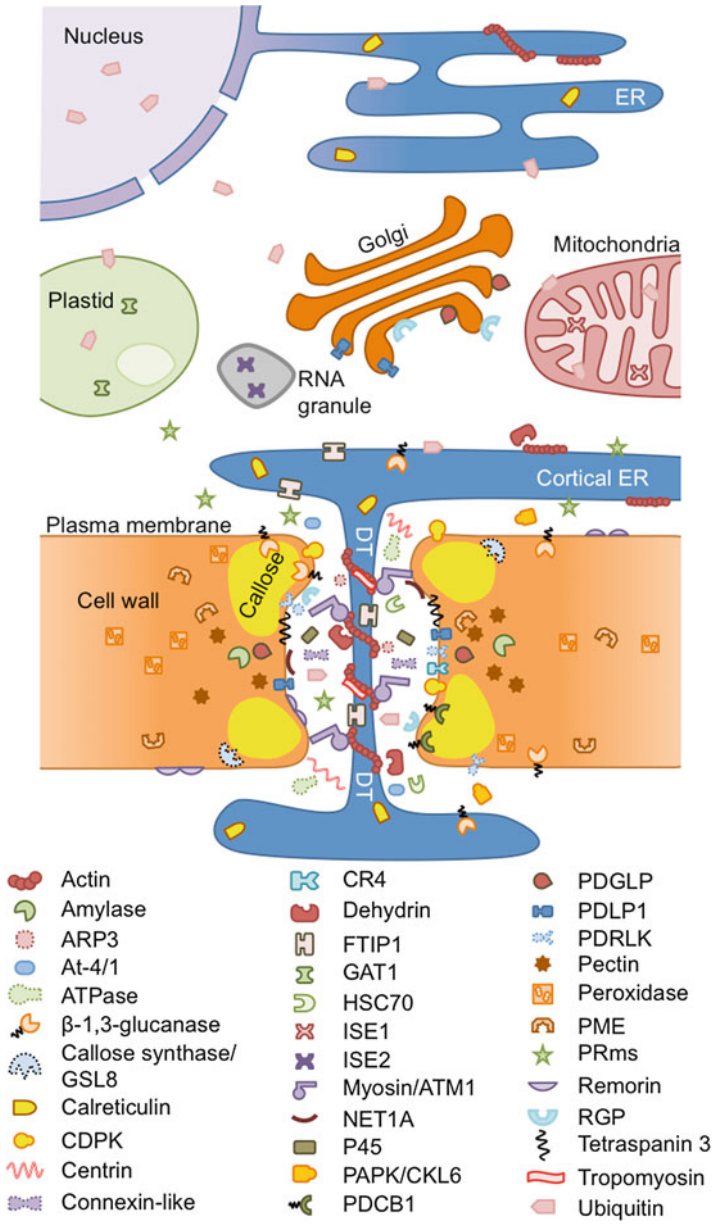

Fig. 8.1 Macromolecular components of plasmodesmata: summary of the proteins and carbohydrates in and around plasmodesmata and their experimental localisation elsewhere in the cell (if known). Refer to Table 8.1 for more information. *Dotted* shape outline means that a firm localisation is not known. Diagram not to scale. *ARP3* actin-related protein 3, *ATM1 Arabidopsis thaliana* myosin I, *CDPK* calcium-dependent protein kinase, *CKL6* CASEIN KINASE-LIKE 6, *CR4* CRINKLY4, *DT* desmotubule, *ER* endoplasmic reticulum, *FTIP1* FLOWERING LOCUS T-INTERACTING PROTEIN, *GAT1* thioredoxin m3, *GSL8* GLUCAN SYNTHASE-LIKE 8, *HSC70* heat shock cognate 70-like protein, *ISE* INCREASED SIZE EXCLUSION, *NET1A*

plasmodesmatal permeability as measured in a GFP diffusion assay and increased callose accumulation in pit fields (Levy et al. 2007), providing further evidence of plasmodesmatal association. The PD callose-binding protein 1 (PDCB1; Simpson et al. 2009) has also been found; like AtBG_ppap, PDCB1 is a glycophosphatidylinositol (GPI)-anchored protein, and yellow fluorescent protein (YFP) fusions were localised at cell wall puncta and were immunolocalised using TEM to plasmodesmatal neck regions in Arabidopsis. Functionally, PDCB1 overexpression mutants accumulated more callose and exhibited decreased PD SEL as measured with the GFP diffusion assay (Simpson et al. 2009). Taken together, these findings again implicate callose as a major regulator of PD aperture, and these proteins no doubt only provide a glimpse of the callose regulatory machinery at PD.

Another carbohydrate-related protein found at PD is α-amylase. This enzyme was immunolocalised to barley aleurone PD using TEM and possibly functions in wall hydrolysis preceding more extensive endosperm hydrolysis (Gubler et al. 1987). In addition, the wall carbohydrate, pectin, has been immunolocalised using fluorescence microscopy and TEM to pit fields in fruit pericarp (Casero and Knox 1995; Roy et al. 1997; Orfila and Knox 2000) and *N. tabacum* trichomes (Faulkner et al. 2008). Pectin methylesterase (PME), a pectin modifying enzyme, has also been immunolocalised to plasmodesmata using TEM as well as to the middle lamella and plasma membrane of flax hypocotyls (Morvan et al. 1998; Chen et al. 2000). PME has been shown to be a target for MP^{TMV} binding and may facilitate viral cell-to-cell movement (Dorokhov et al. 1999; Chen et al. 2000).

8.2.1.2 Cytoskeletal Elements

A number of cytoskeletal proteins thought to function in regulating plasmodesmata (White and Barton 2011) have been located in PD. Actin (White et al. 1994; Blackman and Overall 1998) and myosin-like (Blackman and Overall 1998; Radford and White 1998) and centrin-like (Blackman et al. 1999) proteins have been immunolocalised using TEM to plasmodesmata, actin and myosin being found along the entire length of plasmodesmata and centrin predominantly near the orifice region. Additionally, an Arabidopsis class VIII myosin ATM1 (*A. thaliana* myosin 1) has been localised to plasmodesmata (Reichelt et al. 1999; Baluška et al. 2001; Golomb et al. 2008; Sattarzadeh et al. 2008). The physical arrangement of actin and myosin within PD remains a subject of conjecture (Tilsner et al. 2011; White and Barton 2011). Since actin filaments are associated with cortical ER tubules (Quader et al. 1989; Boevink et al. 1998), it is conceivable that actin would be associated with the

Fig. 8.1 (continued) NETWORKED 1A, *PAPK* plasmodesmata-associated protein kinase, *PDCB1* plasmodesmata callose-binding protein 1, *PDGLP* plasmodesmata germin-like protein, *PDLP1* plasmodesmata-located protein 1, *PDRLK* plasmodesmata-associated receptor-like kinase, *PME* pectin methylesterase, *PRms* pathogenesis response-related protein from maize seed, *RGP* reversibly glycosylated polypeptide

desmotubule (Blackman and Overall 1998). Overall and Blackman (1996) proposed that it could be the protein that forms a spiral-like structure around the desmotubule membrane (Fig. 8.1). However, the recent discovery of Networked 1A (NET1A), an actin-binding, plasma membrane-associated protein, at PD (Deeks et al. 2012) challenges this model. It may be that actin filaments are associated or even partially embedded within the plasma membrane of PD.

In support of a PD localisation and function, *N. benthamiana* myosin VIII was found to be necessary for the PD localisation of a movement protein from *Beet yellow virus* (Avisar et al. 2008), and antibodies against Arabidopsis myosin VIII increased cell-to-cell transport of fluorescent dyes when microinjected together into Arabidopsis root epidermal and *N. tabacum* leaf mesophyll cells (Volkmann et al. 2003).

Unsurprisingly, proteins that interact with these cytoskeletal elements have also been detected in plasmodesmata. The actin-related protein 3 (ARP3) has been immunolocalised to pit fields (van Gestel et al. 2003), and a tropomyosin-like protein appeared to immunolocalise along the length of PD (Faulkner et al. 2009). Although microtubules have not been detected in PD (Blackman and Overall 1998; Aaziz et al. 2001), a GFP fusion with the Arabidopsis microtubule-associated protein 65-5 (MAP65-5) was observed to remain across nascent post-cytokinetic walls, which was interpreted as potential PD localisation (van Damme et al. 2004).

8.2.1.3 Protein Kinase and Calcium-Related Proteins

Since PD regulation appears to be closely linked to ATP levels, phosphorylation (Blackman and Overall 2001; Oparka 2004) and calcium, it is fitting that a number of protein kinases and calcium-related proteins have been found at plasmodesmata. An *N. tabacum* plasmodesmata-associated protein kinase (PAPK) was isolated from plasmodesmata-enriched protein fractions (Lee et al. 2005); however, since clones could not be identified due to sequence conservation, an Arabidopsis ortholog (CASEIN KINASE LIKE6; CKL6) was cloned and localised to or near plasmodesmata by coexpression with GFP-tagged MP^{TMV} (Lee et al. 2005; Ben-Nissan et al. 2010). Functionally, PAPK and CKL6 were found to selectively phosphorylate a number of NCAPs, including MP^{TMV} and LFY but not KN1 (Lee et al. 2005). This is significant as phosphorylation of MP^{TMV} is thought to affect its ability to increase plasmodesmatal aperture (Waigmann et al. 2000). Protein kinases with putative signal transduction functions have also been localised at plasmodesmata. Maize CRINKLY4 (CR4), a receptor-like kinase, has been immunolocalised specifically to maize aleurone PD using TEM (Tian et al. 2007). CR4 is most likely involved in cell fate determination of aleurone cells; in fact, PD connecting adjacent cells in the aleurone exhibit increased SEL, effectively forming an aleurone symplasmic domain (Tian et al. 2007). Other PD-associated receptor-like kinases have been isolated from rice and appear to co-localise with *Turnip vein-clearing mosaic virus* MP at PD (Jo et al. 2011). An autophosphorylating calcium-dependent protein kinase (CDPK) has been immunolocalised to punctate spots in maize mesocotyl cell

walls and is found in PD-enriched protein extracts (Yahalom et al. 1998). In keeping with the role of calcium in regulating PD, calreticulin, a calcium-binding chaperone resident in the ER lumen, has been immunolocalised to plasmodesmata in maize root tips using fluorescence microscopy and TEM (Baluška et al. 1999) and also in Arabidopsis using TEM (Christensen et al. 2010). In fact, it seems that calreticulin has started to become a *bona fide* plasmodesmatal marker (e.g. Bayer et al. 2006; Faulkner et al. 2008). In addition to calcium and phosphorylation, ATP levels also play a role in regulating plasmodesmata—reduced ATP levels enhances cell-to-cell transport (Tucker 1993; Christensen et al. 2009; Radford and White 2011). Consistent with this, ATPase activity has been cytochemically detected at plasmodesmata (Cronshaw 1980; Chen et al. 1994), especially around plasmodesmatal orifices (Belitser et al. 1982).

8.2.1.4 Membrane Related

Since PD contain plasma membrane and endoplasmic reticulum, it is not surprising a number of membrane-associated proteins have been found there. Early efforts to immunolocalise proteins to PD leveraged the similarity between the plant channels and animal gap junctions, successfully labelling punctate cell wall spots and PD with antibodies to animal connexins, the structural components of gap junctions (Meiners and Schindler 1989; Meiners et al. 1991; Yahalom et al. 1991; Schulz et al. 1992). However, subsequent sequence analysis has disputed the nature of these antigenic plant proteins, instead identifying them as protein kinase-like proteins (Mushegian and Koonin 1993). Later efforts have involved purification of PD-enriched protein fractions (reviewed in Faulkner and Maule 2011) successfully finding a number of membrane-associated PD proteins such as PDCB1 (Simpson et al. 2009). In the same screen that produced PDCB1, the PD-located protein (PDLP) family was uncovered, having members that localised to PD upon fusion to GFP (Thomas et al. 2008). Functionally, overexpression mutants of PDLP1 exhibited reduced PD permeability as measured by GFP diffusion; the converse was observed in double knockout mutants of redundant PDLP family members (Thomas et al. 2008). PDLPs have also been recently proposed to function as receptors for the MPs of tubule-guided viruses (Amari et al. 2010). Significantly, the transmembrane domain of plasma membrane-associated PDLP1 was found to be necessary and sufficient for PD targeting (Thomas et al. 2008). Indeed, fluorescently tagged PDLP1 has seen use as a PD marker (e.g. Raffaele et al. 2009), although like the use of MP^{TMV} as a marker, ectopic expression of fluorescently tagged PDLP1 could have deleterious effects on PD and may cause aberrant false-positive or false-negative localisations, especially given that transgenic Arabidopsis overexpressing tagged PDLP1 are stunted and exhibit dramatically reduced PD permeability (Thomas et al. 2008). In another screen of plasmodesmata-enriched wall fractions, a 41 kDa wall-associated protein was discovered (Epel et al. 1996), which was later identified as a class 1 reversibly glycosylated polypeptide (^{C1}RGP), a family of peripheral membrane proteins (Sagi et al. 2005). A GFP fusion of an Arabidopsis ortholog,

AtRGP2, localised to plasmodesmata (Sagi et al. 2005). Functionally, TMV spread is limited in *N. tabacum* overexpression mutants (Zavaliev et al. 2010); however, the plants are severely stunted possibly due to general stress-induced plasmodesmatal occlusion from overexpression.

Interest in membrane rafts, also known as lipids rafts, in plants has been growing recently. Membrane rafts are specialised membrane functional and structural domains, and there is mounting evidence of their presence in plasmodesmata (Tilsner et al. 2011)—membrane rafts are typically enriched in GPI-anchored proteins, of which PDCB1 and β-1,3-glucanase are examples. Remorin, a likely component of plant membrane rafts, has been immunolocalised to plasmodesmata in tomato and *N. tabacum* using TEM. It was suggested to inhibit viral cell-to-cell movement since it was observed that *Potato virus X* lesions were smaller in plants overexpressing remorin and larger in plants with lower remorin levels (Raffaele et al. 2009).

8.2.1.5 Other Plasmodesmatal Macromolecules

Many other PD-associated proteins are known and their functions are varied. Through mutagenesis screens of Arabidopsis either exhibiting increased or decreased symplasmic transport fluorescent probes, three proteins have been identified: INCREASED SIZE-EXCLUSION LIMIT 1 and 2 (ISE1 and ISE2; Kim et al. 2002; Kobayashi et al. 2007; Stonebloom et al. 2009) and GFP ARRESTED TRAFFICKING 1 (GAT1; Benitez-Alfonso et al. 2009). Although not technically PD-localised proteins (ISE1 is found in mitochondria, ISE2 in cytosolic granules and GAT1 in plastids), their influence on PD is noteworthy. For example, *ise1* and *ise2* Arabidopsis mutant embryos and *N. benthamiana* leaves ectopically silenced for ISE1 and ISE2 displayed slightly increased numbers of branched plasmodesmata compared to wild type (Kobayashi and Zambryski 2007; Stonebloom et al. 2009; Burch-Smith and Zambryski 2010), while *gat1* mutants also exhibited slightly elevated numbers of branched and occluded plasmodesmata in root tissues (Benitez-Alfonso et al. 2009). Ectopic silencing of ISE1 and ISE2 in *N. benthamiana* leaves using viral-induced gene silencing leads to increased cell-to-cell movement of GFP-tagged MP^{TMV} (Stone et al. 1984; Burch-Smith and Zambryski 2010), and ectopic expression of GAT1 in mature Arabidopsis leaves increased transport of monomeric GFP (Benitez-Alfonso et al. 2009). ISE1 and ISE2 encode putative DEAD and DEVH box RNA helicases, respectively, and ISE1 mutants exhibit increased ROS accumulation (Stone et al. 1984; Kobayashi et al. 2007). Interestingly, a protein with strong homology to an Arabidopsis DEAD box RNA helicase has also been identified from plasmodesmata-rich tissue in *Chara* (Faulkner et al. 2005). GAT1 encodes a thioredoxin presumed to be involved in redox homeostasis; indeed, GAT1 mutants over-accumulated ROS (Benitez-Alfonso et al. 2009). Taken together, these studies suggest that ISE1, ISE2 and GAT1 regulate cell-to-cell trafficking through regulation of PD development and perhaps function, possibly through differential responses to redox in different organelles or tissues (Canto and Palukaitis 1999;

Benitez-Alfonso et al. 2009; Burch-Smith and Zambryski 2010). Consistent with a role for ROS at PD, peroxidase activity has also been detected in isolated apple cell walls (Ingham et al. 1998).

The chaperone heat shock cognate 70 (HSC70) has been isolated from pumpkin cDNA libraries after an antibody against the plant heat shock protein 70 (HSP70) family immune-reacted positively with PD-enriched wall fractions (Aoki et al. 2002). Two pumpkin HSC70 proteins were found to increase PD SEL and mediate their own cell-to-cell transport. Functionally, they may assist the refolding of misfolded proteins after transit through PD (Aoki et al. 2002). A maize pathogenesis-related protein (PRms) has also been observed to move cell to cell and has been localised to plasmodesmata using GFP fusions and immunogold TEM (Murillo et al. 1997; Murillo et al. 2003). A subsequent study has shown that PRms is phloem-mobile (Ma and Peterson 2001), and it is suggested to function in pathogen resistance (Murillo et al. 2003). Abiotic stress is also related with plasmodesmatal proteins—a dehydrin-like protein has been immunolocalised using TEM to plasmodesmatal orifices of cold-acclimated *Cornus sericea*, possibly functioning in protection of plasmodesmatal membranes from freezing-induced dehydration damage (Karlson et al. 2003). Two proteins of largely unknown function have also been identified at plasmodesmata: a 45 kDa protein immunolocalised primarily to the central cavities of *Chara* PD (Blackman et al. 1998) and an Arabidopsis protein named At-4/1 which localises to cell wall puncta when fused to GFP (Paape et al. 2006). Finally to close off this section, ubiquitin has been localised to PD where it is thought to be involved in targeting components for degradation since immuno-labelling concentration is highest in plasmodesmata at intermediate stages of degradation (Ehlers et al. 1996).

8.2.2 Architecture of PD

While there has been steady progress in identifying the molecular components of PD in recent years (Fig. 8.1, Table 8.1), there has been almost no advance in our understanding of the structural details of PD. Recent images of PD ultrastructure (see Sect. 3.2) are essentially identical to those shown in Gunning and Robards (1976) yielding little new insights into the arrangement of components within the PD. The difficulty is that the PD diameters are in the order of 50 nm, thinner than the plastic sections used in TEM. This means that in longitudinal images of PD, the information from the entire PD may be superimposed making resolution of the details difficult. In cross section, at least one-third of the length of a plasmodesma will be superimposed so that resolution of the detailed structure is obscured (Overall 1999). There also remains the challenge of distinguishing solid structures from cytoplasmic space based purely on patterns of electron density and electron lucent regions.

In recent years, two novel approaches have been tried in an attempt to gain new understanding about the three-dimensional architecture of PD. High-resolution scanning electron microscopy provides clear images of the distribution of PD

(Faulkner et al. 2008; Brecknock et al. 2011), and in the thick walls of the giant-celled alga *Chara*, it was possible to fracture through the wall to expose the internal structure of these PD (Brecknock et al. 2011). These fractured PD yielded tantalising images of a central structure surrounded by a ring of globules: the technique has been unable to generate the three-dimensional information that is needed. The second approach has been to exploit electron tomography that involves collecting images of the specimen at incremental angles that are then used to generate to digitally generate a three-dimensional model of the structure that can be used to generate thin slices through the object providing clarity not possible with the information superimposed in a resin section (Baumeister et al. 1999). Electron tomograms have been published for plasmodesmata in maize aleurone cells (Tian et al. 2007) and chilled leaves (Bilska and Sowinski 2010). Interestingly both showed undulations in the width of the PD down its length, similar to tomograms of PD produced in our laboratory (Howard and Overall, unpublished) suggesting that the cell wall sleeve around PD can accommodate, or even bring about, changes in the dimensions of PD. Electron tomography promises to provide unparalleled three-dimensional information about PD and should now be exploited, in combination with freeze-substitution, to examine a variety of PD and PD in different physiological states.

8.2.3 Towards a Functional Model of the Macromolecular Architecture of Plasmodesmata

There are a plethora of models of the macromolecular architecture of PD, but they are based almost entirely on conjecture. For example, Overall and Blackman (1996) have developed a model in which the layer immediately outside the ER within PD is depicted as actin that forms a spiral down the length of PD and the electron-dense spokes that radiate out from this layer to the plasma membrane as myosin. While Overall (1999) has presented a reasoned argument as to why this proposed localisation is consistent with TEM images, more than a decade on, there has been no definite proof that this arrangement actually exists inside PD. In fact the recent identification of Networked 1A, an actin-binding protein associated with the plasma membrane (Deeks et al. 2012), would suggest that the actin inside PD is most likely to be closely associated with the cell membrane, not the ER (see Sect. 8.2.1.2). There have been a variety of structures seen in TEM images, often after the inclusion of tannic acid in the fixative (e.g. Overall et al. 1982; Badelt et al. 1994), that have been proposed to be putative sphincters, both internal (see Sects. 1.5.2 and 3.2) and in the wall sleeve surrounding the PD (see Sect. 3.2). There are obvious functions for such sphincters in the regulation of intercellular transport; in fact, the extracellular spirals encircling PD observed by Badelt et al. (1994) are well placed to generate the constrictions along the lengths of PD seen in electron tomograms of PD. Despite the potential importance of these structures in modulating PD

transport, and the developing list of PD proteins thought to be involved in regulation (Table 8.1), there is still no molecular identity for these structures. The only exception is the putative sphincter linking ER and cell membrane at the necks of PD identified through the use of tannic acid (Overall et al. 1982), which has been proposed to be the calcium-sensitive contractile protein centrin (Blackman et al. 1999) on the basis of immunogold localisation to the neck regions of PD. However, even this may not be a consistent feature of PD given that Bilska and Sowinski (unpublished) were unable to localise centrin to PD (see Sect. 3.3.2.2).

Once a three-dimensional structure of PD has been generated, it will remain to localise the macromolecules identified in Fig. 8.1 along with others still to be identified to the structures. The traditional approach of immunogold cytochemistry used to localise the components to PD (Table 8.1) has limited use as the antibodies are only able to bind to antigens on the surface of the plastic resin of the TEM section. It will be necessary to develop antibodies that can penetrate the resin or develop a pre-embedding labelling approach. Alternatively, since the localisation of a number of proteins to PD has relied upon localisation at fluorescent fusion proteins, one approach suggested by Jahn et al. (2012) would be to produce an electron-dense deposit from the fluorescent proteins, such as occurs in photo-oxidation, so that it can be visualised within the PD at high resolution. This technique is yet to be developed for use in plant cells (Jahn et al. 2012). Such an approach would allow correlative microscopy of PD with the powerful combination of live cell imaging of fluorescent tags, useful in identifying the physiological state of PD, followed by specific localisation at high resolution. Super-resolution microscopy (Bell and Oparka 2011) has the potential to generate useful images of PD components, such as ER, actin and myosin, each tagged with different fluorescent markers, at sufficient resolution to distinguish their relative arrangements. Super-resolution microscopy followed by electron tomography in a correlative microscopy study of the same PD would be challenging but could yield the information needed to build a model of PD with the molecular components identified.

The ultimate goal is to develop a macromolecular model of PD that includes functional information. A systematic analysis of the macromolecular structure of PD in mutants of PD proteins or functions or possibly following physiological manipulation could generate such information.

8.3 Rapid Regulation of PD Transport

There is now a solid body of work on the long-term regulation of intercellular communication at the level of blocking of PD or indeed production of new PD, as well as medium-term regulation of the permeability of PD (see Chaps. 1, 2 and 3). However, other than Holdaway-Clarke et al. (2000) who demonstrated rapid reversible closure of PD in response to high cytoplasmic calcium, almost nothing is known of about rapid regulation of PD permeability. This situation has arisen because of the popularity among experimentalists of using the rate of movement of different

sized tracer molecules or large expressed fluorescent proteins such as GFP to assess the permeability of PD. These approaches have the advantage that they are relatively easy to perform and can indicate changes in the size of molecules that can readily pass through PD. However, a major disadvantage of using them is that they have poor time resolution and are unable to detect rapid, particularly reversible, changes in permeability in PD. The only technique currently available able to detect rapid changes in the permeability of PD is the more technically demanding electrophysiology. It is unfortunate that in the last decade, there have been no publications using electrophysiological approaches to study intercellular communication in plants.

There is now an new opportunity to combine the advances from molecular biology in the last decade in the identification of novel PD proteins with electrophysiological approaches to tease out which components of PD are responsible for the rapid regulation of PD permeability by studying specific RNAi knockout or mutant plants. For example, does the rapid reversible increase in permeability of plasmodesmata observed by Holdaway-Clarke et al. (2000) occur in mutants of Arabidopsis lacking centrin or in cells in which the expression of centrin has been downregulated?

8.4 Homogeneity of Plasmodesmata in a Wall

Questions raised by Robards (1976) about the pathway of transport through individual PD in a wall largely remain unanswered today. Does an individual PD transport in only one direction at a time? Are all PD functional at a given time? Are there subpopulations of PD that transport larger molecules and do these preferentially close as the size of molecules that can pass from cell-to-cell decreases rather than all PD in a wall decreasing the size of molecule they can transport? Our recent observation that the ER lumen forms an intercellular transport pathway (Barton et al. 2011) raises the question as to whether transport takes place through the ER and cytoplasmic pathways simultaneously within the one PD and if so whether in both directions or opposite directions in each channel. The techniques used to date to monitor PD transport can only provide information about the net transport across the PD in an entire wall, not at the resolution of individual PD. In order to understand the mechanism of regulation of intercellular transport, the field will need to develop an approach that can provide information about the operation of single PD.

Estimates of PD electrical resistance indicate that they are 400 times more electrically resistive than would be expected if they were simple saline-filled tubes (Overall and Gunning 1982). This estimation was based on the assumption that all PD in a wall showed equal permeability, so a likely explanation for this discrepancy is that only a fraction of the PD are actually functional at one time. Indeed, once technology was available to study the permeability of individual gap junctions (Spray et al. 1991), it became clear that only a fraction of gap junctions were

functional at one time. Another approach to answer some of the questions posed above would be to follow the movement of fluorescently tagged molecules in the ER or cytoplasm by correlative microscopy to identify the position of these molecules within the PD of a wall at high resolution. It has been shown that net transport across an individual wall may be in both directions or polarised (see Sect. 2.2) and that ER and cytoplasmic transport can take place across an individual wall (Guenoune-Gelbart et al. 2008)—this correlative microscopy approach could determine if this occurs at the level of individual PD or if there are subpopulations of PD with specialised function within individual walls.

8.5 Conclusions

Despite the considerable progress that has been made in identifying components of PD, we are still largely ignorant of their structural and functional details. To some degree, progress has been limited by the predominant use of easier technologies in preference to the more challenging approaches that are required to answer the questions remaining. For example, localisation at PD is often confirmed on the basis of co-localisation with PD markers such as callose or other PD proteins at the level of confocal microscopy without confirmation of localisation at the PD at the TEM level. Currently the most popular approach to study transport through PD is to follow the movement of GFP, even though the time resolution of this approach is poor and it only provides information about the transport of large molecules. The challenge for the field now is to invest in the development of new approaches such as correlative microscopy or combine techniques such as electrophysiology with newly available molecular tools to work towards the ultimate goal of a functional model of the macromolecular architecture of PD.

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Index

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