

## 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).

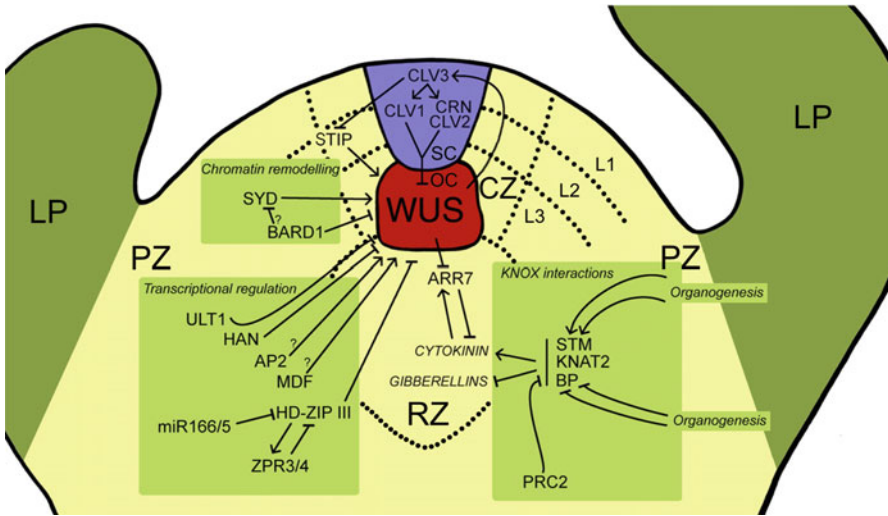
The plant's cell connections, called plasmodesmata, interconnect the cytoplasm of adjacent cells to a symplasmic continuum. Plasmodesmata are even more sophisticated than the analogous structures in animals, called gap junctions, although both types of cell connections function as communicating junctions. 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*,

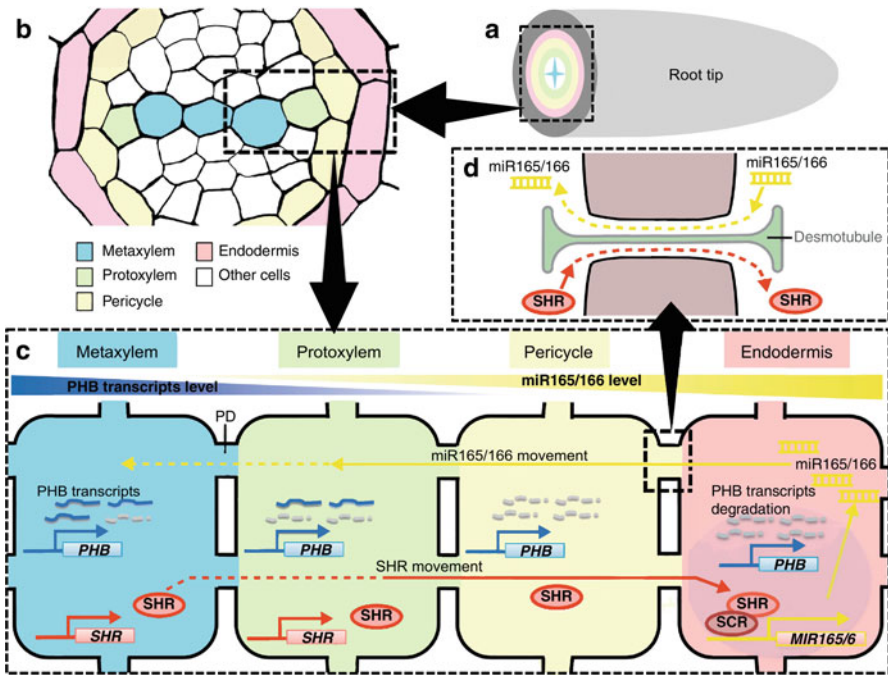


**Fig. 2.1** The molecular network of SAM regulation in *Arabidopsis*. CZ: central zone organized in three clonally distinct cell layers (L1–L3), LP: leaf primordia, OC: organizing center, PZ: peripheral zone showing fast cell proliferation and initiation of lateral organs, RZ: rib zone providing the 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-ZIP III 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

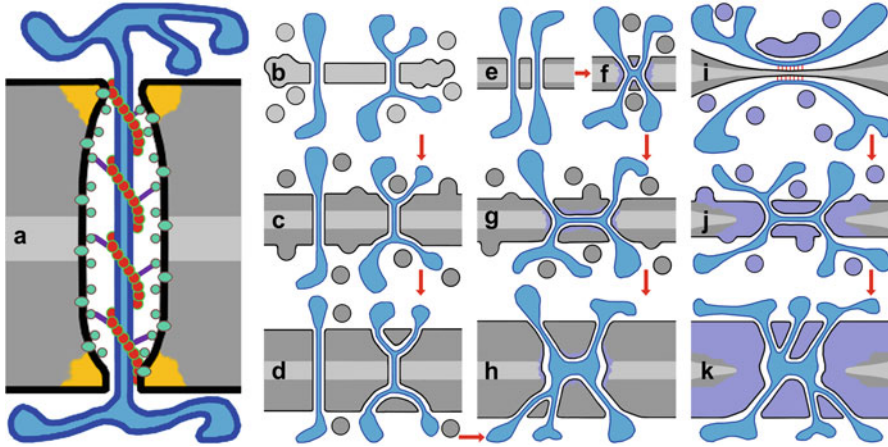
growing plant tissues, including embryos, leaves, roots, and shoots during primary and secondary growth, as well as during the transition to flowering (reviewed, e.g., in Ehlers and Kollmann 2001; Burch-Smith et al. 2011a). The dynamics of the developing plasmodesmata networks facilitate modulations 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. 2.4i 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 corraling,” 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  $\text{Ca}^{2+}$ -dependent protein kinases have been localized at plasmodesmata and may cause alterations of the size of the plasmodesmal microchannels, possibly in a  $\text{Ca}^{2+}$ -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  $\beta$ -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 Zambryski 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  $\beta$ -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.

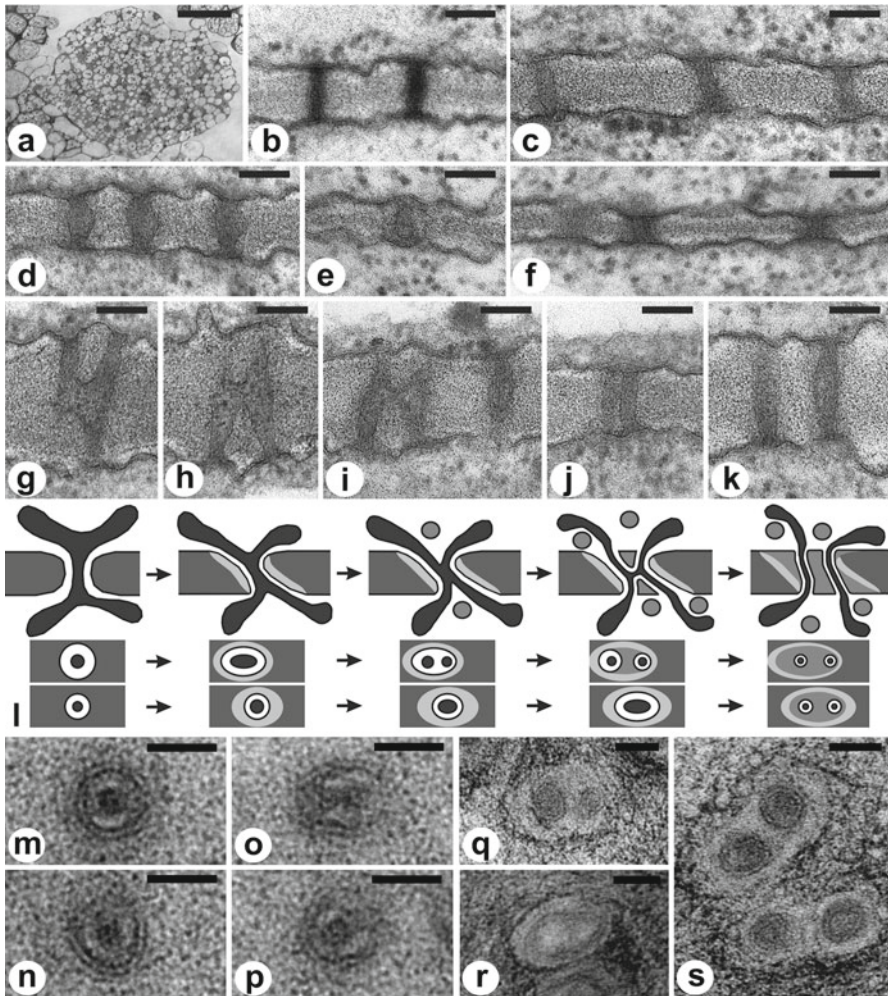
## 2.4 Plasmodesmal Morphotypes and the Mechanisms of Plasmodesmata Formation, Modification, and Deletion

### 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  $\mu\text{m}$  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 “twinning 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 intercellular 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  $\mu\text{m}^2$  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 pre-existing 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; Gisela 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<sub>4</sub> (Rinne et al. 2011), (5) to treatments with sodium azide and N<sub>2</sub> inducing anaerobic stress and a reduction of the cytosolic ATP levels (Tucker 1993; Cleland et al. 1994; Christensen et al. 2009), (6) to H<sub>2</sub>O<sub>2</sub> and to the aromatic amino acid tryptophan (Rutschow et al. 2011), (7) to Ca<sup>2+</sup> (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 C<sub>4</sub> 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  $\mu\text{m}$  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  $\mu\text{m}$  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  $\mu\text{m}^2$  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  $\mu\text{m}^2$  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).

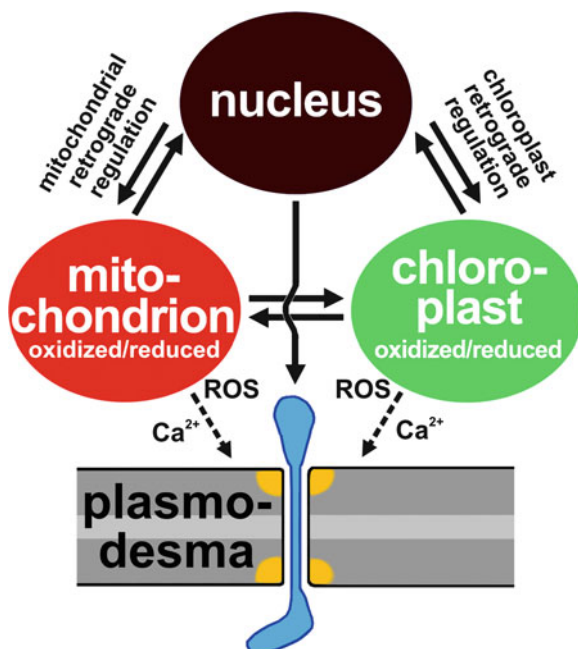
## 2.5.2 Molecular Mechanisms Controlling Plasmodesmal Formation, Modification, and Deletion

### 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  $\text{Ca}^{2+}$  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 *isel1* (*increased size exclusion limit*, Stonebloom et al. 2009, 2012). However, *isel1* has an increased plasmodesmal SEL.

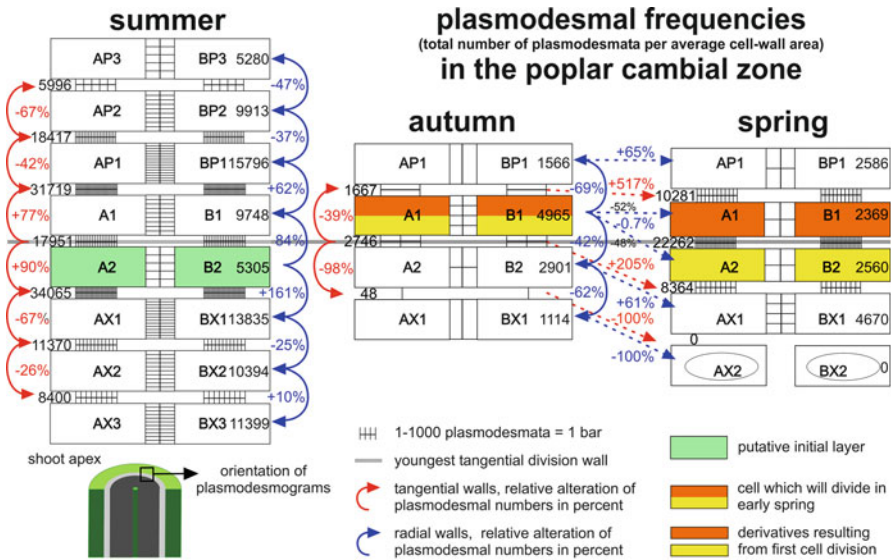
These contradictory results might be explained by different amounts of ROS production in *gat1* vs. *isel1*. Low ROS levels may increase the plasmodesmal SEL, while





**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  $\text{Ca}^{2+}$  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 (buddbreak) 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/AP1) 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. 2.41). 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 ( $\cdot\text{OH}$ ) are generated by class III cell-wall peroxidases from superoxide and  $\text{H}_2\text{O}_2$ , which in turn is produced by a NAD(P)H oxidase in the plasma membrane from the reduction of monovalent  $\text{O}_2$  (Liszkay et al. 2003). The highly active  $\cdot\text{OH}$  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  $\cdot\text{OH}$  in wall-extension growth suggested by Schopfer et al. (Schopfer et al. 2002; Liszkay et al. 2003) has been challenged, because the  $\cdot\text{OH}$  amount needs to be low to avoid damage of the living cells (Cosgrove 2005; Schopfer 2006). Further,  $\cdot\text{OH}$  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  $\cdot\text{OH}$  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  $\cdot\text{OH}$  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 2.4i) 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 2.4i). 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 *rhb2-1*, coding for a NADPH oxidase responsible for ROS production, is epistatic to *rhb3-1* during root-hair growth in *Arabidopsis* (Schieffelin 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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