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Vaidurya Pratap Sahi
František Baluška *Editors*

# Concepts in Cell Biology - History and Evolution

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*This book is dedicated to the memory of Peter W. Barlow (1942–2017)*

# Preface

The conceptualization of this book took place at the first European Cytoskeletal Club meeting held in Prague in June 2015, after a discussion about the need to know the genesis of the concepts that have shaped the science of plant cell biology. Plants have been a subject of study since the times of the ancient Greeks, Chinese, and Indians. Since the first publication of the term "cell" in *Micrographia*, 350 years ago by Robert Hooke (1665), the study of plant cell biology has moved ahead tremendously. Robert Hooke is credited for his observations of cork and for coining the word "cell." In addition to his observations, he inadvertently introduced cell walls and the dynamics of cells in the context of the volume enclosed by the cell walls. Since the days of Hooke, cells, the units of organismal forms, have attracted the attention of the scientific field. The field of cell biology owes its genesis to physics, which, through microscopy, has been vital in enhancing the interests of scientists in the biology of the cell. Today, with the technical advances in the field of optics, it is possible to observe life even at the nanoscale. From Hooke's seminal observation of cells and his inadvertent observation of the cell walls, we have moved forward to engineering plants with modified cell walls. Study of chloroplast has also moved from the experiments of Julius von Sachs to chloroplast engineering for improved crop yields. Similarly, advances in fluorescence microscopy have enabled better observation of organelles, such as the vacuoles studied by Hofmeister.

If physics is one side of the coin (cell biology), biochemistry is the other. We have come a long way from Hooke's observation, but it remains important to understand what Hooke observed 350 years ago, or what Schleiden and Schwan said about cells as the universal units of plants and animals. All this needs to be taken into consideration when we talk about cells, cell walls, or cell cycles. In this digital age, when new techniques are allowing science to move faster than ever, it remains important to bring back old concepts in the light of modern science. In this book, we bring forth and discuss the concepts and theories propounded by the progenitors of plant cell biology in the context of their relevance to the cell biology of today. An understanding of the works of scientists such as Hooke, Grew,

Hofmeister, Schleiden, Schwann, von Mohl, Nägeli, von Mohl, Fleming, Strasburger, Sachs, Bernard, Boveri, Nemec, and Haberlandt in the context of twenty-first century advances in plant cell biology will help to provide new bottles for the old wines. As Isaac Newton wrote to Robert Hooke in his letter dated 5 February 1675: "You have added much several ways, and especially in taking the colours of thin plates into philosophical consideration. If I have seen a little further it is by standing on the shoulders of Giants" (Newton 1675). Newton quoted the twelfth century theologian John of Salisbury, who used it in a treatise on logic called *Metalogicon*, written in Latin in 1159: "We are like dwarfs sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours" (Salisbury 1159). In some way, we are acting as Cedalion standing on the shoulders of Orion in the stories of Greek mythology.

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# Contents

# Chapter 1
# Plant Cell Biology: When, How, and Why?


**Vaidurya Pratap Sahi and František Baluška**

*Julius Sachs (1875) defined cells as follows: "The substance of plants is not homogeneous, but is composed of small structures generally indistinguishable by the naked eye; and each of these, at least for a time, is a whole complete in itself, being composed of solid, soft, and fluid layers, different in their chemical nature, and disposed concentrically from without inwards. These structures are termed Cells."*

## 1.1 When?

About 250 years before Sachs's definition of the cell, Robert Hooke observed cells for the first time on cork. Not only did Hooke's observations start a new wave in the study of plant biology, but it also gave us the term "cell" (Hooke 1665; Gest 2009). The etymological roots of the term lie in the Latin word *cellulae*, which means hexagonal cells of the honeycomb (Mazzarello 1999). Soon after Hooke made his observations and coined the term "cell," Antony van Leeuwenhoek discovered motile microorganisms (Ford 1995; Dunn and Jones 2004; Zwick and Schmidt 2014; Lane 2015; Wollman et al. 2015; Zuidervaart and Anderson 2016). Later, Marcello Malpighi and Nehemiah Grew published detailed observations of the different plant organs and tissues (Malpighi 1679; Grew 1682). Grew described the honeycomb-like cells, but also other forms of cells, which formed the bark and the pith (Grew 1682).

Cell biology has come a long way since the time of Hooke. With the advancements in microscopy (Schliwa 2002), it has become easier to observe other organelles and structures such as cell walls, nuclei, and chloroplasts, and to understand

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the mechanisms of cell division and other processes. In fact, the cell wall can be said to be the first component of the cell to have been observed when Hooke looked at cork cells. The nineteenth century can be said to be the century of cell biology. Discovery of the nucleus, mitochondria, protoplasm, Golgi, etc. and the foundations of cell theory all occurred in the nineteenth century. Studying the leaves of orchids, Robert Brown discovered the nucleus, which he defined thus: "In each cell of the epidermis of a great part of this family, especially of those with membranaceous leaves, a singular circular areola, generally somewhat more opake than the membrane of cell is observable. This areola [nucleus], which is more or less distinctly granular, is slightly convex, and although it seems to be on the surface is in reality covered by the outer lamina of the cell" (Brown 1833). Knowledge of the structure and biology of the nucleus paved the way for a better understanding of genetics and thereby helped in applied sciences such as crop breeding. The discovery of the nucleus was followed by the formulation of cell theory by Matthias Jacob Schleiden and Theodor Schwann (Schwann 1847; Baker 1948, 1949; Harris 2000; Baluška et al. 2012; Lombard 2014; Cvrčková 2018, Sekereš and Žárský 2018).

Hugo von Mohl (1852) gave the term "protoplasma" and described the movement of cell sap (Baker 1949). Protoplasma as defined by von Mohl is "*opake, viscid fluid of a white colour, having granules intermingled it*." The granules he refers to are organelles such as plastids, mitochondria, and Golgi (Benda 1898; Golgi 1898; Mazzarello et al. 2009; Pagliarini and Rutter 2013). It took more than 300 years after the discovery of cells (Hooke 1665) to accept the endosymbiotic nature of eukaryotic cells (Sagan 1967; Baluška and Lyons 2018). In cell biology, organelles or structures were often reported well before they were studied in detail. Bohumil Němec discovered thick filaments running longitudinally across the cell–cell borders in root apices (Němec 1901), which were later shown to be bundles of F-actin (Baluška et al. 1997; Baluška and Hlavačka 2005) extending through plasmodesmata (Šamaj et al. 2006; van Bel 2018). The discovery of chloroplasts was similar. On a general note, plastids were known to the ancient Indians (Raghavendra et al. 2003). The cell plate in dividing plant cells was observed by Wilhelm Hofmeister as early as 1867, when eukaryotic cell division was not well understood (Hofmeister 1867; Martin 2017).

## 1.2 How?

Anthony Leeuwenhoek first discovered the marvels of the living world, which before him were invisible (Ford 1995; Dunn and Jones 2004; Zwick and Schmidt 2014; Lane 2015; Wollman et al. 2015; Zuidervaart and Anderson 2016). Advances in the development of superior lenses by Abbe and Schott in Germany took observations to a higher level of magnification (Schliwa 2002; Dunn and Jones 2004; Blancaflor and Gilroy 2000; Griffiths et al. 2016). The importance of microscopy for physiology was suggested by Jan Evangelista Purkinje in the first half of the nineteenth century (Nick 2012; Žárský 2012). We have come a long way from the first observations of the cell by Hooke to the detailed study of organelles

possible today using the concepts of confocal microscopy (Schliwa 2002; Blancaflor and Gilroy 2000; Griffiths et al. 2016). With advanced microscopy, advances in dyes and probes have also played an important role in enabling detailed study of cells and their components (Blancaflor and Gilroy 2000). Advances in microscopy have enabled us not only to see the organelles but also to observe and understand the interactions between them (Griffiths et al. 2016).

In addition to advances in microscopy, microtomy, and histochemistry, the ability to grow cells has allowed fast progress in our understanding of plant cells. Haberlandt (1902) is credited to be the first to have cultured plant cells in nutrient solutions. Vasil (2008) reviewed the history of plant cell culture and its use in biotechnology. Cell parameters such as size, shape, and number can be studied in cell cultures and used for cellular phenotyping (Opatrný et al. 2014).

## 1.3 Why?

Cell biology is sometimes considered to be a basic science, which is not true because of the implications it has in applied fields such as medicine and agriculture (Nick and Chong 2012; Vasil 2008). The study of plant cells has not only enhanced our understanding of plants, but also made it easier to tap plant resources for purposes such as crop biotechnology. Nick (2012) suggests how we can take cell biology to a new level by integrating the conceptual knowledge of nineteenth century cell biologists (botanists) and modern high-throughput tools.

Another important aspect is that scientific concepts are very important for proper interpretation of obtained data and even more important for asking the right questions and choosing an appropriate research methodology and experimental design. By guiding so-called normal science, scientific concepts shape emerging and maturing paradigms until new concepts lead to revolutionary overthrow of the old paradigm and raise new emerging paradigms (Kuhn 1962; Guerra et al. 2012; Kaiser 2012; Casadevall and Fang 2016). For example, the concept of spontaneous generation of life was proposed by Aristotle and dominated our thinking about life until Louis Pasteur disproved this theory (Pasteur 1864; Berche 2012). Recently, there have been several paradigm shifts in genetics (Portin 2015), plant sciences, and neurosciences (Baluška and Mancuso 2009a, b; Trewavas and Baluška 2011; Trewavas 2016; Calvo et al. 2017); evolutionary theory (Shapiro 2011; Baluška 2011); and in the basic pillar of cell biology, cell theory (Baluška et al. 2004a, b; Baluška and Lyons 2018). First attempts to understand the nature of consciousness and its roles in biology (Trewavas and Baluška 2011; Baars and Edelman 2012; Perouansky 2012; Grémiaux et al. 2014; Rinaldi 2014; Baluška et al. 2016; Torday and Miller 2016; Torday 2017; Craddock et al. 2017) and physics (Baars and Edelman 2012; Turin et al. 2014; Craddock et al. 2017) indicate that a new paradigm shift is imminent. Many crucial discoveries in cell biology were accomplished through study of plants, including discoveries of the cell, nucleus, the symbiotic origin of eukaryotic organelles, microtubules, cell–cell channels,

chromosomes, mitosis, and the cell cycle (Baluška et al. 2012; Lombard 2014; Cvrčková 2018, Sekereš and Žárský 2018). As higher plants are proving to be behaviorally active and cognitive organisms (Baluška and Mancuso 2009a, b; Sahi et al. 2012; Grémiaux et al. 2014; Trewavas and Baluška 2011; Trewavas 2016; Calvo et al. 2017), and their active behavior to be sensitive to anesthetics (Grémiaux et al. 2014; Baluška et al. 2016; Yokawa et al. 2017), it is possible that plants will also prove to be crucial for our understanding of the elusive nature of consciousness.

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# Chapter 2
# 180 Years of the Cell: From Matthias Jakob Schleiden to the Cell Biology of the Twenty-First Century


**Juraj Sekeres and Viktor Zarsky**

**Abstract** The fact that the form and function of organisms results from the collective action of cells, the structural and functional units of life, is undoubtedly one of the most important foundations of contemporary biology. Here, we provide a glimpse of the key discoveries and accompanying theoretical disputes that led from the discovery of the cellular structure of organisms, through elaboration of a tool set enabling study of cell phenomena at the molecular level in a mechanistic framework, to the latest theoretical and methodological trends in addressing cellular organization as the methodological and interpretational framework for addressing the phenomena of life. We also emphasize how views of cell structure and function prevailing during particular eras were influenced by methodological constraints at the time and how previously disregarded concepts returned to mainstream biology as a result of novel techniques that could provide more detailed insight into the structure and dynamics of cellular components.

## 2.1 Theoretical and Methodical Foundations of Cell Theory

Various ideas, first in the form of mythological narratives, on the origins and basis of life have existed since the dawn of humankind. Some of the first modern ideas on the substance of life and its developmental program being confined to a small piece of living organism came from Aristotle in the fourth century BC. On the basis of empirical experience of egg development and plant vegetative reproduction, he postulated "entelchy" as a driving principle that leads organisms toward fulfilling their form and potential (Welch and Clegg 2010). Until the seventeenth century, the

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developing science of biology only rarely sought the causes of live phenomena in the fine structure of organisms. Prominent trends such as French morphology and German *Naturphilosophie* looked for explanation of body plans and structures in abstract ideal forms toward which organisms are driven (Radl 1930); the approach was largely orthogonal to later and contemporary mechanistic views of life.

Idealistic concepts accompanied biology further, but a gradual shift toward empiricism and mechanistic tendencies in science appeared in the eighteenth century. The trend included revival of atomism, a theory that claims that properties of matter are given by the small indivisible particles it is composed of (Harris 2000). It is important to note that advances resulting in formulation of cell theory were not only led by technological improvements in microscopy, but also by a change in theoretical focus. Many scholars already had the idea that observed tissues were aggregates of more basic units, even before looking through microscopes (Harris 2000). Another important philosophical inspiration (quite distinct from common early analogies between cells and atoms or crystals, and much closer to the contemporary perception of cells) came from G.W. Leibniz (1646–1716). His idea established the often unrecognized basis of cell theory. In the idea of fully autonomous self-reproducing "monads," developed in a critical discourse with the Cartesian mechanistic view of the universe, Leibniz stated that if living organisms were machines their parts would not merely be simple mechanical pieces of matter but smaller machines themselves. Importantly, the dynamics of monads is driven from the inside. This idea stimulated the concept of German philosopher Lorenz Oken (1779–1851) that all organisms are composed of "infusoria" and "Urbläschen" (primordial bubbles) as basic life units; this speculation directly preceded the works of the first empirical cell biologists (Canguilhem 2008; Harris 2000). However, it was only the invention and improvement of microscopes that enabled direct observation of the material basis and composition of organisms.

Based on early observations, the composition of tissues as fibers, globules, or twisted cylinders was postulated (Harris 2000). In the eighteenth century, Albrecht von Haller, inspired by atomism, speculated that fibers composed of strings of atoms were the basic structural elements of the body: "For the fiber is for the physiologist what the straight line for the geometrician, and from this fibre all shapes surely arise" (in Harris 2000). Robert Hooke was active in many fields of natural sciences in the second half of the seventeenth century and is considered to be the father of the term "cell" in biology. He used this term to describe the structures he saw with his simple microscope in slices of plant cork tissue because they resembled honeycomb cells (*cellulae* in Latin). At that time, cells were conceived as hollow and regarded as "avenues of communication, channels for conveyance of juices" (in Welch and Clegg 2010).

## 2.2 From Schleiden to Virchow: Formation of Cell Theory Tenets

More and more nineteenth century scientists were convinced that plant tissues were generally composed of cells, but Matthias Jakob Schleiden (1804–1881) made the first attempt to use cellular composition as a unifying explanatory principle in botany (Harris 2000). Schleiden wanted to establish botany on a firm ground as a more exact science, leaving behind the speculative tradition of German *Naturphilosophie*. Schleiden was mechanistically oriented and, like many of his contemporaries, inspired by Isaac Newton's physics. He used crystal-like metaphors for conceptualizing the self-organization of organisms. He also emphasized inductive and empirical approaches, as well as the importance of following ontogeny (reflecting specification or differentiation of initial simpler general forms into more complex elaborated ones) in order to properly understand plant tissues. Schleiden's efforts resulted in the formulation of a general rule that all plant tissues are composed of a single basic element, the polyhedral cell. He would subsequently call for "condemnation of every theory that explains processes in a plant otherwise than as combination of processes in individual cells" (in Radl 1930).

The cell wall as the boundary and structural element was still considered more important than the internal content, although the nucleus was already known and described. It was named in 1833 by Robert Brown who, however, did not recognize the general presence of nuclei in all cells. Such an opinion is understandable considering that the cell wall is morphologically the most conspicuous structure in differentiated plant cells and is often the functional determinant of the particular tissue. Moreover, the crucial importance of cell wall mechanics and its integration with the plant cell membrane and cytoplasmic core are currently well-recognized features of plant body organization. Schleiden was unclear about the ontogenic origin of cells; therefore, an extracellular protoplasm or sap played a role in his concept of cell formation. He postulated condensation of nuclei from this material and formation of cellular matter around them. Cells were formed from nuclei as growing vesicles until they touched each other (Harris 2000; in Radl 1930). Later in development, they mostly formed around nuclei inside other cells (Lombard 2014). Schleiden considered nuclei in mature cells dispensable and often reabsorbed (Harris 2000).

Because of the absence of distinct cell walls and difficulties in sample preparation, the cellular nature of animal bodies was less clear. Animal cells were studied, for example in developing embryos. However, the general empirical supposition postulated formation of the animal body from cells during early development, but not necessarily in its adult state. Henri Dutrochet (1776–1847) advocated a materialistic worldview and aimed to identify vital phenomena in animals and plants (Harris 2000). He claimed that both plant and animal tissues were composed of "vesicles" and "globules," although he probably could not observe animal cells. Although Dutrochet's morphological view of cells was largely erroneous, he was probably the first to perceive cells as basic physiological units of metabolic

exchange with selective inflow of nutrients and outflow of waste. He also suggested the existence of the same underlying principles in animal and plant tissues: "[If] phenomena are tracked down to their origins, the differences are seen to disappear and an admirable uniformity of plan is revealed" (in Harris 2000).

The first claim of the widespread presence of "Kornchen" analogous to plant cells in animal tissues, backed by countless histological observations, was made by Bohemiam Jan Evangelista Purkyně/Purkinje (Harris 2000). He claimed that animal tissues were universally composed of cells, fibers, and fluids. Purkyně was also one of the first (following Dutrochet) to emphasize the functional significance of cells and facilitated the transition from "histomorphology" to "histophysiology" (Harris 2000), particularly through comprehensive studies of ciliary movements in several animal tissues. Unlike Schwann, who put most emphasis on the nucleus, Purkyně also focused on the active content of the cell, the "protoplasm" (see Sect. 2.3).

Theodor Schwann (1810–1882) got most credit for extending cell theory to animal tissue because he made stronger (although not always correct) claims than Purkyně (Harris 2000). Inspired by Schleiden's conclusions, as well as the similarity between animal notochord cells and plant cells discovered by Schwann's teacher Johannes Müller (Harris 2000), Schwann accumulated a vast number of examples of embryonic and adult animal tissues consisting of cells and claimed cellular origin as the unifying ontogenic principle for animals as well as plants (Radl 1930). Schwann was not certain about the exact origin of individual cells and postulated their origin either from homogenous life matter (possibly through first generating a nucleus) or from inside other cells, around their nuclei. According to Schwann, cells could thus originate inside or outside other cells (Harris 2000; Lombard 2014). Inspired by Schleiden, Schwann claimed that formation of cells from liquid via nuclei was a mechanistic crystallization-like process (Harris 2000).

Several different ideas about the mechanism of new cell generation coexisted and many scientists accepted that different mechanisms could work in different organisms and tissues (Harris 2000). Discovery of binary cell fission by Barthélemy Dumortier and Hugo von Mohl was of outstanding importance, although both admitted the plurality of mechanisms of cell formation. Franz Unger (1800–1870) was the first to oppose Schleiden's aggregation/crystallization idea openly. He disregarded "cytoblasts" as source of cells and postulated that binary division was the most common mechanism of plant cell division (Harris 2000). Within a few years, sufficient empirical evidence had accumulated to abandon Schleiden's concept of cell formation. Because of technical difficulties, it took much longer to accumulate precise observations of animal cell formation. Robert Remak (1815–1865) proposed the first explicit unifying theory of cell division in both plants and animals. Remak developed novel hardening agents that allowed him to carry out extensive studies of cell formation in many animal tissues. He concluded that extracellular formation of cells does not occur in animal tissues and that binary division is the universal mechanism of cell formation. Development is thus a sequence of binary divisions followed by morphological modifications; furthermore, the egg itself is a cell. Remak also proposed that the same rules governed cell

division in both pathologic and embryonic tissues (in direct opposition to Müller's theory of specific malignant tumor formation). Remak categorically opposed Schleiden and Schwann, particularly their analogies between cells and crystals: "It is hardly necessary to make special mention of the similarity or disparity of cells and crystals, for, in the light of the facts that I have discussed, the two structures offer no points of comparison" (in Harris 2000).

Rudolf Virchow (1821–1902), strongly inspired by Remak and spreading Remak's ideas, consolidated cell theory with his famous statement "*Omnis cellula e cellula*," reflecting the origin of existing cells from other cells and describing ontogeny as a gradual process of binary divisions from a fertilized egg to adult tissues. Classical cell theory thus stood on three major tenets:

1. All living organisms are composed of one or more cells.
2. The cell is the basic unit of structure and function in all organisms.
3. All cells arise from preexisting cells.

The history of discoveries leading to a unified picture of cell division (and the relationship between nucleus and cytoplasm during formation of new cells) is an excellent example of how the choice of methods and model system can influence the inferred theory. This aspect has always constrained experimental biology and is still relevant in our time. From the contemporary point of view (i.e., retrospective judgement), ideas about extracellular formation and crystallization around nuclei might seem obscure. However, one must acknowledge that many conclusions were based on observations of fixed tissues prone to artifacts and providing only a static view of underlying dynamic phenomena. The presence of open mitosis in both animals and plants (Sazer et al. 2014) made deciphering the relationships between "sap" (cytoplasm), nucleus, and cell division even more complicated until the nature of chromosomes was understood.

Moreover, some of the tissues used in the past as model systems are nowadays known as rather exceptional cases. Even original observations of plant tissues by Schleiden involved endosperm syncytium undergoing cellularization, which might have given him the wrong impression of cell formation (Harris 2000). Many early conclusions were also misled by mistaking starch grains (forming inside cells) for nuclei. On the other hand, Dumortier and von Mohl were able to make their outstanding discovery through observing an ideal model system for study of binary cell division—the filamentous alga *Conferva (Draparnaldia by contemporary nomenclature)* with cells dividing at the termini of filaments. Cartilage was repeatedly used as argument for the acellular origin of animal cells (Harris 2000). Developing embryos, which enabled direct observation of unfixed dividing cells in time, were the source both of support for a model of binary cell division and of erroneous judgment. Although many authors (working mostly with amphibian models) correctly interpreted the partitioning of egg as progressive cell division, French biologist Quatrefages de Bréau claimed in the middle of the nineteenth century that the development of gastropod embryos involves formation of cells within cells. Quatrefages de Bréau was probably driven by an attempt to support Schwann's model. Even Dumortier, who discovered binary division in *Conferva*

*(Draparnaldia by contemporary nomenclature)*, acknowledged the possible formation of cells within cells and even formation of cells from acellular material after observing gastropod development (Harris 2000). Large yolky embryos with unequal cleavage were also source of confusion, as in the case of Carl Vogt who claimed that *Alytes* frog embryo furrowing was independent of formation of new cells.

In the light of incongruent fragmentary observations, it was honest of many contemporary scientists in the nineteenth century to acknowledge the plurality of animal cell formation mechanisms (Harris 2000). Strong universal claims required systematic comparison of many different tissues and improved techniques, as performed by Remak. Although he opposed ideas that involved intracellular formation of cells, he admitted that it was often not sloppiness of observation or ill judgment that lead to incorrect conclusions, but accidental choice of problematic material such as cartilage or muscle fiber. However, even Remak made an erroneous conclusion regarding nuclear division, possibly because of observation of static fixed specimens and a bias toward making an analogy between binary cell division and binary nuclear division. Karl Bogislaus Reichert (1811–1883) observed dissolution of nuclei during division of red blood cells, which he used as an argument for Schwann's concept of de novo nuclei formation and against the concept of binary cell division. Remak claimed that he had failed to reproduce Reichert's observation of nucleus dissolution in dividing red blood cells. Reichert's ideas about cell formation were generally wrong but some of his observations were correct, whereas Remak's ideas about cell formation were generally right but some of his observations were wrong. Remak occasionally observed nuclear dissolution but interpreted it as an artifact. Both Remak and Virchow supported a model of nuclear binary division that involved formation of grooves, constriction, and division of one nucleus into two. Some scientists advocated Remak's and Virchow's models, whereas others referred to nuclear dissolution ("Reichert's doctrine"), often with interpretations close to Schwann's original ideas about cell formation (Harris 2000).

## 2.3 Protoplasmic Concepts and Early Criticisms of Newly Established Cell Theory

Cell theory was popular with reductionists, who attempted to comprehend fundamental life phenomena by studying simple structural components. Technological improvements such as the oil immersion lens, Purkyně's microtome technique (Harris 2000), and novel fixation and staining methods (McIntosh and Hays 2016) led to countless observations of cells and their contents in the nineteenth century. Criticism of cell theory also existed and, in extreme cases, many histological discoveries were accused of being staining and/or fixation artifacts. Skepticism over the universality of cell theory often cited the existence of cells without nuclei,

multinuclear syncytia, and large amounts of extracellular material in adult tissues as evidence against cell theory. Nevertheless, all of these phenomena were ultimately understood as developmental products of cells. One of the last bitter arguments about the general validity of cell theory was over the nature of nervous tissue. "Reticulate theory" considered the nervous tissue as a continuous uninterrupted network, because of observation limits set by contemporary microscopes. Yet, cell theory envisaged nervous tissue as consisting of individual cells as in other tissues (the "neuronal doctrine"). Ramon y Cajal demonstrated the latter to be true by using a staining method that randomly marked only a few neurons within the tissue, clearly indicating discontinuity in the neuronal network (Radl 1930).

In addition to claims that cell theory cannot universally explain the functioning of organisms and that many observed structures might be fixation artifacts, cell theory was also repeatedly accused of being insufficient or even not relevant to understand the universal properties of life. Some of these incongruences were formulated in various forms of "protoplasmic theory," which either complemented cell theory by closing a conceptual gap between the cell surface and cellular contents or competed with cell theory by completely shifting focus from cells as a mere building bricks to the living substance inside the cell. The term "protoplasm" was introduced by Jan Evangelista Purkyně/Purkinje in 1839, well before Hugo von Mohl and in a very similar sense (Janko and Štrbáňová 1988; Harris 2000; Zárský 2012; Liu 2016). Hugo von Mohl was critical of Schleiden's and Schwann's focus on understanding cells in terms of boundaries and building blocks and disliked analogies between cells and crystals. He redefined the cell's function as more based on internal organization and formulated his protoplasmic theory in 1846 (Liu 2016).

Ferdinand Cohn proposed in 1850 that "plants and animals were analogous not only because of their construction from cells, but also, at a more fundamental level, by virtue of a common substance, protoplasm, filling the cavities of those cells" (Welch and Clegg 2010; Liu 2016). He thus connected von Mohl's concept with the earlier idea of "sarcode," a contractile substance proposed by Félix Dujardin to provide the life basis of unicellular eukaryotes (Liu 2016). The tendency to look for basic attributes of life (irritability, sensibility, contractility, reproduction, etc.) in the properties of protoplasm was not uncommon, and protoplasm itself was compared to an "elementary organism." Anatomist Max Schultze suggested in the middle of the nineteenth century that the true basis of life would be found by studying protoplasm, not the cell (Welch and Clegg 2010) and redefined the cell as a "clump of protoplasm" around a nucleus (Liu 2016).

Some authors regarded the cell as a nonliving envelope and focused on studying protoplasm as the "naked state of living matter" (Welch and Clegg 2010). For example, E.B.Wilson did not claim protoplasm to be the only living element inside the cell: "Protoplasm deprived of nuclear matter has lost, wholly or in part, one of the most characteristic vital properties, namely, the power of synthetic metabolism, yet we still speak of it as 'living', because it may for a long time perform some of the other functions, manifesting irritability and contractility, and showing also definite coordination of movements" (as in the enucleated protozoan) (Wilson

1899). He also disregarded strong versions of reductionism that searched for a single basic element of life in "any single substance or structural element of the cell," because "life in its full sense is the property of the cell-system as a whole rather than of any one of its separate elements." His theory is thus not atomistic or reductionistic but puts a strong focus on the properties of protoplasm by claiming "that the continuous substance is the most constant and active element and that which forms the fundamental basis of the system, transforming itself into granules, drops, fibrillae or networks in accordance with varying physiological needs" (Wilson 1899). Yet, Wilson prophetically admitted that he could not achieve any clear general conclusion because the basis of all phenomena lies in the "invisible organization of a substance which seems to the eye homogenous." He believed that "ultramicroscopic bodies," molecules, groups of molecules, and micellae formed the basis of protoplasmic organization (Wilson 1899).

## 2.4 Discovery of Organelles: Increasing Appreciation of Cellular Content

Along with protoplasmic concepts involving the actions of micelles, drops, and tiny fibrillae, the presence of larger structures localized within cells was more and more recognized and emphasized, including the notion of smaller living units present inside cells, inspired by Leibnitz's theory of spontaneity and hierarchy of monads (see Sect. 2.1). Franz Unger described moving structures in pollen cytoplasm as an "army of monads full of inner vitality, full of an inner self-determination that revealed itself in their movements" (in Harris 2000). Observations of large unicellular eukaryotes such as amoebae and ciliates further stimulated thoughts about subcellular structures with specialized functions, analogous to macroscopic bodies. In 1884, Karl August Mobius suggested the term "organulum" (little organ) for such structures because they form parts of one cell, whereas true organs of multicellular animals consist of many cells. The term was later transformed into "organelle" and its meaning was expanded to cover subcellular structures of both unicellular and multicellular organisms (Schuldiner and Schwappach 2013).

An important breakthrough was made by van Benden and Boveri at the end of the nineteenth century. They discovered the autonomous life cycle of the centrosome and concluded that the structure had a life of its own; Boveri described the centrosome as a special organ of cell division (Harris 2000). Whitman perceived the cell as a "colony of simpler units, nucleus, centrosome, and so on," much as a higher organism is colony of cells (Whitman 1893). In 1882, Julius Sachs wrote that "chlorophyl bodies" (chloroplasts) behaved like autonomous organisms that divide to adjust their number to the size of growing leaves (Kutschera and Niklas 2005). In 1883, Andreas Schimper noticed the similarity between chloroplasts and cyanobacteria and proposed the symbiotic cyanobacterial origin of plastids (Taylor 1987). In 1890, Altmann postulated the universal presence of "bioblasts" (named

"mitochondria" by German microbiologist Benda in 1898) and discovered that they had same staining properties as bacteria; he concluded that they were modified bacteria (Ernster and Schatz 1981; Kutschera and Niklas 2005).

This idea of the endosymbiotic origin of chloroplasts and xenobiotic origin of eukaryotic cells as an evolutionary amalgam of once-independent organisms was further elaborated by Konstantin Mereschkowsky between 1905 and 1920 (Taylor 1987; Kutschera and Niklas 2005), but was not generally accepted until its revival in the 1970s. With improved microscopes and staining methods, novel organelles were added to the nuclei, chloroplasts, and vacuoles known from earlier observations (Ernster and Schatz 1981). With the discovery of "ergatoplasm" (later named "endoplasmic reticulum") in 1897 and the Golgi apparatus one year later, most large common components of the cell "inventory" were known by the end of the nineteenth century (Ernster and Schatz 1981).

## 2.5 Disputes over Cell Boundaries

For a living system, the existence and properties of a boundary to the outside world are as important as the properties of its internal composition. Yet, the presence and identity of a boundary between cells and the outside environment was not clear in the nineteenth century and (especially from the contemporary perspective) was largely neglected by proponents of both cell and protoplasmic points of view. Schwann assumed that surfaces/membranes always limit the mobility in/out of a cell, even if invisible, and this could be inferred from the Brownian motion of cell components, which do not escape the cell volume as delimited by the surface structure. Generally, however, comparison of the cell surfaces of plant cells (with walls) and animal cells were confusing and the terms "wall" and "membrane" were often used interchangeably. True membranes were impossible to detect with nineteenth century histology techniques. Thus, in the second half of the nineteenth century, little attention was paid to membranes and, if present, they were considered unessential secondary structures originating from hardening of the cell surface. Max Schulze, the proponent of protoplasmic theory, was also an eager opponent of the membrane concept (Lombard 2014). He postulated, in place of cells, small blebs of contractile protoplasm immiscible with water. Detected membranes were simply the result of protoplasm hardening caused by contact with the outside environment or an artifact of degeneration and the hallmark of dead cellular material.

The main support for the membrane concept came from osmotic studies. Hewson published experiments on the swelling and shrinking of blood cells as early as 1773. In the first half of the nineteenth century, Dutrochet explained plant turgescence by osmosis via a border with "chemical sieves" (Harris 2000; Lombard 2014). The first artificial membranes were created by precipitation of copper ferrocyanide (from potassium ferrocyanide and copper sulfate) and were thus named precipitation membranes. Together with the contemporary colloidal concept

of cell interiors and ideas about cell membranes originating through surface hardening, the existence of artificial precipitation membranes fueled belief that the surface of colloidal protoplasm precipitates and forms an osmotic barrier. Overton's pioneering experiments (published between 1895 and 1900) showed cell volume changes in more than 500 different solutions and allowed him to conclude that a barrier distinct from the plant cell wall must exist and is made of ether-soluble components (i.e., is hydrophobic). He suggested cholesterol and phospholipids as possible candidates. In combination with works on electrophysiology and microinjection experiments, acceptance of the plasma membrane as a real structure was established in the early twentieth century (Harris 2000; Lombard 2014).

## 2.6 Toward Cellular Determinants of Heredity

A clear picture of nuclear division formed only after the mitotic spindle and chromosomes were discovered and understood. Recurrent observations eventually led to the consensus that nuclei disassemble and reassemble during cell division. Strassburger proposed homology of plant and animal cell division before the end of the nineteenth century (Harris 2000). In the 1870s, details of cell division events were repeatedly observed and, in 1879, Walter Flemming coined the term "mitotic process" and described its basic chronology. Flemming also introduced the term "chromatin" and was the first to describe longitudinal division of chromosomes in both animal and plant cells. He was a sharp critic of the direct nuclear division concept advocated by Remak and Virchow, but at the same time fully acknowledged the continuity of nuclear material during cell division by expanding Virchow's statement into "*Omnis nucleus e nucleo*."

At that time, there was also a major effort to localize the material determinants of heredity. Many great biologists of the nineteenth century, even if not working with cells themselves, postulated such particles (Darwin postulated gemulae; Haeckel, plastiduls; Spencer, physiological units; de Vries, pangenes; Galton, strips, etc.) and thus stimulated the search for them (Radl 1930). Cumulative descriptive work helped characterize the progression of cell division and behavior of chromosomes in sufficient detail that biological interpretations and manipulative experiments were possible. As early as 1885, the concept of chromosomal loops as storage place for hereditary information was proposed by A. Weissmann (McIntosh and Hays 2016) and helped to explain the phenomena of meiosis and recombination (Harris 2000). The work of Theodor Boveri (1862–1915) not only definitively demonstrated chromosome function in heredity, but also shifted work from solely combination of observations and deduction to the introduction of manipulative experiments (Harris 2000). His experiments with sea urchin embryos involved polyspermy and manipulation of early embryo cleavage, resulting in blastomeres with unequal chromosome distribution. Boveri discovered that the fate of blastomeres correlated with introduced chromosomal abnormalities and deduced that different chromosomes carry different genetic loads. After the rediscovery of

Mendel's laws, Boveri was the first to point out the similarity between segregation of elements, as proposed by Mendel, and physical segregation of chromosomes (Harris 2000). The first concept of genes was purely phenomenological and did not necessarily ask for the material agent of heredity. Later, in the light of mechanistic trends, a material component responsible for transmission of genetic information was envisaged. Boveri proposed that the material basis of Mendel's laws of inheritance lay in the properties of chromosomes and thus contributed to the development of molecular genetics in the twentieth century (Harris 2000).

## 2.7 Cells in Tissues: Early Holistic and Reductionist Experimental Approaches

Since the early days of cell theory, many scientists have stressed that organisms are more than just an assembly of their parts, and that functional aspects of life should be studied in the context of the whole developing embryo/organism. Attitudes ranged from sharp criticism of cell doctrine as insufficient and misleading, through attempts to introduce novel organizing principles that would supplement and coordinate the action of cells, to a systematic attempt to understand developing embryos purely from the collective interactions of individual cells.

T.H. Huxley put forward a physiological interpretation of the cell in opposition to Schleiden's and Schwann's morphological concept. He claimed that "the cell-theory of Schleiden and Schwann" was not only "based upon erroneous conceptions of structure," but it also led "to errors in physiology" (Richmond 2000). He particularly disliked that "cell doctrine" overstated the assumption of anatomic individuality of cells and felt that cells should be studied in their mutual relation in the context of development, because the entire life history of an organism is "dominated by development" (Richmond 2000). Whitman stated that "the fact that physiological unity is not broken by cell-boundaries is confirmed in so many ways that it must be accepted as one of the fundamental truths in biology" (Whitman 1893). Sachs advocated the organism-standpoint and considered the presence of cells, although a general phenomenon of life, to be of secondary importance and only one of the many manifestation of formative life forces (Whitman 1893). The idea of Sachs that growth and change of plant forms is primary and that planes of cell division are secondary and dependent on overall growth (Radl 1930) was also shared by de Bary, who coined the famous statement: "The plant forms cells, the cells do not form plants" (Thompson 1917).

Major attempts at causal analysis of embryonic development as a result of collective interaction of individual cells crystallized into the discipline of *Entwicklungsmechanik* (developmental mechanics in the sense of natural causation), enthusiastically advocated by Wilhelm Roux (Radl 1930; Sander 1991). Roux shifted focus from speculations based purely on descriptive observations to manipulative experiments in a quest for causal explanation of development by

combination of individual acting forces (Priven and Alfonso-Goldfarb 2009; Sander 1991). Based on his experiments with amphibian embryos, Roux advocated a mosaic concept of development, stating that cells of the early embryo determine the position of later parts of the organism.

Other scientists proposed different concepts of development, largely because they used other model systems, such as cnidarians and early developing embryos that display an astonishing capacity for regeneration and a certain degree of invariance of morphogenesis with respect to the number of cells participating. Such experiments suggested that cells of the same lineage can have different fates and cells of different lineages the same fate, depending on the position they acquire within the embryo. Whitman claimed that "Comparative embryology reminds us at every turn that the organism dominates cell-formation, using for the same purpose one, several, or many cells, massing its material and directing its movements, and shaping its organs, as if cells did not exist, or as if they existed only in complete subordination to its will" (Whitman 1893). Some of the trends even resulted in the search for holistic principles that precede formation of cells and organize actions of cells across the whole developing organism.

Hans Driesch also attempted to break the continuous process of animal morphogenesis into its ultimate elements (first principles) at the outset of his career (Sander 1992a). In a visionary manner, he considered development to "start with a few ordered manifoldnesses," which would gradually "create, by interactions, new manifoldnesses," which "acting back upon the original ones (manifoldnesses) provoke new differences." "With each response, a new cause is immediately provided, and a new specific reactivity for further specific responses." (Sander 1992a). Parts of the developing embryo thus constitute a gradual conversion of states and receptivity to other stimuli. Governed by the nucleus, organogenetic chemicals are formed in the cytoplasm, which acts as intermediaries between external stimuli and the nucleus. A cascade of stimuli between cells and their partial activations drive development of the organism (Sander 1992a). Later in his life, Driesch became critical of overestimating the explanatory potential cell theory (Whitman 1893) and even revoked some of his original positions (Sander 1992b). Experiments with cnidarians, acrasid slime molds, plants, and echinoderm embryos (Markoš 2002; Sander 1992b) led him to search for fundamental laws determining the spatiotemporal coordinating system that leads cells into form (Priven and Alfonso-Goldfarb 2009; Sander 1993). Driesch advocated a mathematical and physical approach (Priven and Alfonso-Goldfarb 2009) but also wanted biology to be a science with autonomy and thus searched for organization principles, around which the undergoing chemical and physical phenomena are constituted (Priven and Alfonso-Goldfarb 2009). His conclusion that contemporary chemistry and physics were not sufficient to explain embryogenesis could in fact be extended until the 1970s, when cell research incorporated advances in cybernetics and genetics (Roth 2011). Driesch put strong emphasis on teleology in development (Sander 1992b) and unsuccessfully tried to formulate entelechy as a new collective physical quantity (Markoš 2002; Priven and Alfonso-Goldfarb

2009), specific for organisms, which might be analyzed using mathematical approaches (Priven and Alfonso-Goldfarb 2009).

Driesch's attempt to uncover laws of organization typical for biology was further developed by Alexander Gurwitsch (Beloussov 1997; Markoš 2002). Gurwitsch studied developing shark brain, fungal fruiting bodies, and composite flowers and arrived at the general conclusion that the overall shape repeatedly develops in an exact manner despite fluctuations in the shape and growth rate of individual parts. He also thought that the outline of a part or a whole embryo can be formulated mathematically more precisely than the shape and arrangements of their internal components (Beloussov 1997). Looking for a supracellular principle that orders and coordinates cells over the embryo, and inspired by contemporary developments in physics, he formulated the concept of a "species-specific field" that organizes morphogenesis (Beloussov 1997; Markoš 2002). Cells produce the field that extends to and affects an extracellular space and, at the same time, the field acts back on the cells. Fields from cells form an aggregate field, which depends on the configuration of the multicellular whole and there is feedback between the field and its morphogenetic consequences (Markos 2002). The interdependence between cell properties and their coordinates of position within a developing organism should be precise and mathematically simple (Beloussov 1997). Gurwitsch even attempted to define the field in vectorial manner (as a geometric description, not in a strictly physical sense), where cells followed the vectors of the field (Markoš 2002).

By the 1930s, many crucial discoveries in experimental embryology had been accomplished. Many studies involved isolation and recombination of embryonic parts and mapping of the differentiation and inductive potential of the isolated parts of embryos and the effects of parts transplanted onto other embryos, including interspecific transplants (Oppenheimer 1966; Gilbert et al. 1996). Phenomena such as the inductive potential of neural folds and establishment of limb polarity were intensively studied. Hans Spemann reintroduced the term "field of organization" to describe the inductive properties of the amphibian dorsal blastopore (Gilbert et al. 1996), conceptually building upon Driesch's concept of a "harmonious equipotential system." The concept of a field was thus still vital and, in 1939, Paul Weiss postulated that field is the key organizing principle of embryology; developmental phenomena have field properties and components of fields are connected by a web of interactions (Gilbert et al. 1996). Field concepts in the 1930s experimental embryology were materialistic. Weiss claimed that field has physical existence and is bound by physical substrates from which morphogenesis arises and should be the object of research like any other physical phenomena. The morphogenetic field was supposed to become the basic paradigm of embryology in its attempt to discover the laws of morphogenesis (Gilbert et al. 1996).

## 2.8 Establishment of Molecular Biology

Details of the birth and early history of biochemistry are beyond the scope of this review. However, we mention several key discoveries and concepts because the paradigm and methodology elaborated by biochemists largely influenced the advent of modern cell biology, especially in the twentieth century. Although most German scientists studying cells focused on their structure and formation, the French naturalist Francois Vincent Raspail (1794–1878) was interested in the chemistry of cells. He analyzed the chemical composition of cells by adopting chemical combustion analysis for small samples (microburning) and developed staining procedures to detect starch, albumin, silica, mucin, sugar, chlorides, and iron. He also stressed that that the cell is itself a microlaboratory, carefully balancing catabolism and anabolism (Harris 2000). In 1833, Payen and Persoz purified a thermolabile fraction able to breakdown starch into sugar. Such "agents" were later named enzymes by Wilhelm Kuhne. In 1893, Eduard Buchner was able to replicate the whole yeast fermentation process by a cell-free extract. Thomas Burr Osborne systematically crystallized proteins and demonstrated a vast diversity of protein species (Kyne and Crowley 2016). In 1926, James Sumner managed to isolate and crystallize an enzyme (urease) for the first time. He redissolved urease from the crystal (thus free of any small compounds potentially co-purified from the cell) and showed its catalytic activity, also demonstrating the proteinaceous (and biopolymer) nature of enzymes (Quastel 1985; Kyne and Crowley 2016).

The initial approach of biochemistry was thus orthogonal to that of microscopy. The properties of life would be studied outside of the organismal context, irrespective of the structural principles in the intact body. The aim was to replicate life or life-like processes in an isolated system with a minimal set of components and thus isolate the underlying substances in order to understand the ongoing properties and changes of matter. Parts of the "protoplasmic" concept were dropped or overshadowed by the advent of classical biochemistry, which focused on isolated molecules in buffered water solutions of simple composition (Kyne and Crowley 2016). The simplified "bag of enzymes in solution" perception of cell content, where molecules randomly encounter each other and follow the law of mass action, was criticized at the outset of the science of biochemistry. It was suggested that catalytic agents act as part of an integral and dynamic proteinaceous network in the cell. However, the original focus of early biochemistry on enzymes as catalytic agents provided a unified mechanistic tool set for characterizing subsets of cellular components and phenomena (Welch and Clegg 2010; Kyne and Crowley 2016). Molecular biology is currently understood as based on molecular genetics, but before the ability to modify genetic information was acquired, it was biochemistry that established the first true molecular-level reductionist description of some life processes.

Synthesis of Mendelian and chromosomal heredity theories in the early twentieth century put genes into the spatial context of location on chromosomes and stimulated institutionalization of genetics as a discipline. As a result of the

successful reductionist approach and the immediate economic impact on breeding, there was a common tendency to put genetics into the center of a mechanistic biology framework (Gayon 2016). For example, developmental genetics arose as an alternative program that competed with established experimental embryology (instead of being proposed as a complementary approach). Both the concept of gene used by geneticists and the concept of field used by embryologists were abstract and both were considered to have a physical basis, although understood only vaguely. At that time, genes were still considered to be associated with the action of proteins, possibly enzymes (Oppenheimer 1966; Gilbert et al. 1996). Genocentric tendencies were thus evident in biology at least two decades before the tenets of molecular biology were consolidated. The concept of field as an organizing principle was eventually abandoned, largely because biochemical techniques to examine field phenomena in detail were not available, whereas techniques for study of gene expression in model systems gradually appeared (Gilbert et al. 1996). Despite continuous attempts to interpret life in a holistic framework or perspective, reductionist approaches prevailed in biology as a pragmatic framework for finding mechanistic explanations of complex phenomena.

Genetics, biochemistry, and biophysics developed independently for some time, but started to converge after the 1930s. Key experiments on genetic regulation of *Neurospora* biochemistry in the 1940s showed that each step in a metabolic pathway is controlled by a single gene and this led to the "one gene–one enzyme hypothesis," which suggested that each gene acts directly as an enzyme or determines the specificity of an enzyme (Gayon 2016). This further stimulated perception of the gene as a central unit of biological function and much of the attention turned to the relationship between nucleic acid and protein macromolecules and the search for the molecular basis of heredity. Introduction of novel techniques such as X-ray crystallography and ultracentrifugation helped to turn the focus from colloidal theories to biopolymers and their structures.

Recapitulating the great endeavors of twentieth century molecular biology is beyond the scope of this review and is thoroughly described elsewhere (e.g., Rheinberger 2010). Most importantly, the material basis of hereditary information in the form of nucleotide sequences of nucleic acids was discovered and the genetic code solved, uncovering the relationship between a gene sequence and the protein macromolecule it encodes. Discoveries of the basic principles of molecular biology further stimulated the search for genes responsible for all sorts of processes in living organisms.

With basic metabolic pathways mapped, biochemists became interested in the regulation of metabolism. After the pioneering research of Jacques Monod (1910–1974) on the regulation of biochemical pathways and gene expression (Pardee and Reddy 2003), the concepts of positive feedback, negative feedback, allosteric regulation, cooperativity, induction of enzymes, control by repression, nonlinear regulation, cross-inhibition, and boolean integration of regulatory processes became the standard vocabulary of molecular biology (Monod 1972; Pardee and Reddy 2003). Parallels between molecular biology and cybernetics were thus grounded (Monod 1972), although ideas about cell signaling and gene expression at

the time were rooted in biochemistry and simple cybernetic relations. Newly developed tools shifted the focus onto study of individual genes and their protein products or simple signaling, genetic, and biochemical pathways. It was understood that other components such as extracellular matrix (ECM) components and membrane lipid composition also play important roles (Monod 1972) but, because of technological difficulties, they were neglected in comparison with research performed on DNA and proteins. These molecules were understood to be localized inside cells but more focus was put on understanding their function at a molecular level than on their cellular functions in terms of structural organization of the cells.

## 2.9 Biological Membranes in the Twentieth Century: From Discovery of Lipid Bilayers to the Fluid Mosaic Model

Despite initial neglect of the cell barrier in the nineteenth century, the nature of biological membranes became an important topic in twentieth century cell biology. In 1925, Gorter and Grendel performed a pioneering experiment addressing the structural nature of the plasma membrane. They picked erythrocytes, cells devoid of internal membranes, as the model system and showed that the ratio of monolayer area formed from extracted lipids and erythrocyte surface area was 2:1, suggesting the bilayer nature of the plasma membrane (Lombard 2014). It is noteworthy that the experiment was criticized for several shortcomings, including neglecting the protein components of the plasma membrane and wrong calculation of erythrocyte surface. It is now believed that several experimental errors reciprocally cancelled each other, leading to the correct conclusion. However, the validity of this early model can only be appreciated in the light of much later experiments. Regardless of the criticism, the immediate impact of the lipid bilayer hypothesis was to open discussion on the molecular nature of membrane structure. Trends based on Traube precipitation membranes and Overton lipid membranes were both popular. In terms of molecule permeability prediction, a crucial component of the former was pore size and of the latter, hydrophobicity. The unifying theories assumed membranes to be lipid layers interrupted by pores. The mixed roles of lipids and proteins in the function of membranes were acknowledged, but their relative contribution was a controversial issue (Lombard 2014).

In addition to the iconic character of the search for molecular heredity determinants and solving the differential role of proteins and nucleic acids in the nucleus, another key question in twentieth century cell biology was the nature of protein and lipid interplay in the functioning of biological membranes. Various models involved mixtures of lipid and protein fractions within or between postulated layers of the membrane. Interestingly, one of the concepts dominating membrane research for decades was the "paucimolecular model," which postulated a lipid layer sandwiched between two protein layers. The model was based on measurement of surface tension between echinoderm/teloostei cells and an oil layer, as well as the

structure of myelinized axons. The surface tension experiments were soon criticized for using triacylglycerol instead of native membrane phospholipids, and for using myelinized axons as representative model for a general cell membrane. Nevertheless, the concept became popular for a long time and early low quality electron microscopy (EM) images were interpreted as supporting the paucimolecular membrane model. As in many other cases, a well-intended set of experiments and choice of model system led to wrong assumptions that persisted for decades (Lombard 2014).

Mosaic models of the plasma membrane were also popular. Speculations involving fat-like parts and protoplasmic-like parts, a mixture of sieve-like and solvent elements, were supported by permeability experiments at the beginning of the twentieth century. Permeability experiments also suggested that "pore" diameter could change according to the hydration of the pore, pH, metabolic activity, and cell type but the molecular mechanisms of membrane properties were unclear. Even the breakthrough experiments of Hodgkin and Huxley on membrane excitability (1952) were phenomenological and the mechanism of differential membrane permeability toward $Na^+$ and $K^+$ ions was not known (Lombard 2014). Because hydrated $Na^+$ ions are larger than hydrated $K^+$ ions, selective protein agents facilitating $Na^+$ transport were difficult to imagine. Lipid-based carriers specific for $Na^+$ were postulated. Furthermore, several arguments against the lipid nature of plasma membranes were based on its high water permeability. These conundrums were eventually solved in the context of a delicate structure of the potassium channel and the late discovery of aquaporins, membrane proteins that facilitate water permeability.

The fluid mosaic model dominated the membrane field in the 1970s. It was compatible with most contemporary experiments and predicted future observations; the model remained basically unaltered for next few decades. One of its main advantages over several competing models was compatibility with the thermodynamics of protein–lipid and lipid–lipid binding within membranes, largely based on hydrophobic interactions (Singer 2004; Lombard 2014). The general focus on proteins was fostered by tools developed for molecular biology, resulting in membrane proteins being the primary target of research looking for molecular agents of particular membrane functions. Lipids were considered to be passive structural elements that mostly ensured fluidity of proteins within the membrane. Such an idea is still advocated in many textbooks.

## 2.10 Insights into Cell Ultrastructure and Organelle Origin in the Twentieth Century

The classical descriptive endeavor of cell theory continued during the twentieth century with the disciplines of histology and cytology. The methodological barrier of microscopy was broken in the 1930s by the introduction of electron microscopy.

In combination with novel fixation, sectioning, and staining techniques, it became possible to image subcellular structures with the precision of tens of nanometers. First EM images of mitochondria immediately revealed the presence of a double membrane with inner membrane folds, named cristae (Ernster and Schatz 1981). In 1953, EM helped rediscover the endoplasmic reticulum (Schuldiner and Schwappach 2013). EM not only served as a tool for discovering novel details of subcellular structures, but also brought independent confirmation of conclusions on some older conundrums or questions. For example, several competing models of plasma membrane structure existed and Fischer still opposed membrane theory in 1921, arguing that membranes were invisible even when boundaries of cells were visible (see Sect. 2.9). EM eventually confirmed the presence of a plasma membrane lipid bilayer even in bacterial cells, where its presence had been debated for a long time (Lombard 2014). The generally accepted neuronal theory was also unequivocally confirmed by visualizing the synaptic cleft, a small space between neighboring neural cells. The high spatial resolution enabled detection of novel fine branching structures connecting other cellular components (Welch and Clegg 2010). This microtrabecular network was considered the "basic solid component of cytoplasm," but was also deemed a fixation artifact by many opponents. The concept of solid/liquid phases and heterogeneity of cytoplasm thus became hot topic for some time but then disappeared, only to come back in recent years (Welch and Clegg 2010).

The idea of symbiogenesis (introduced by Mereschkowsky) as the appearance of evolutionary novelties, including novel cell organelles, was revived by Lynn Margulis in the 1970s (Taylor 1987; Chapman and Margulis 1998; Kutschera and Niklas 2005). Margulis also propagated the concept of serial endosymbiosis, stating that modern eukaryotic cells originated by multiple successive symbiogenetic events of once independent organisms (Taylor 1987), and the idea that symbiogenetic events were a common driving force in eukaryotic speciation (Kutschera and Niklas 2005). With employment of molecular biology techniques, support for the endosymbiotic origin of mitochondria and plastids soon accumulated and the paradigm of eukaryotic cell evolution shifted from gradual accumulation of changes as the only mechanism to the possibility of abrupt acquisition of organelles (Taylor 1987). Revival of the symbiogenetic organelle concept and the idea of the eukaryotic cell as a product of cellular fusion between Archea and Eubacteria (Kutschera and Niklas 2005) points to the crucial role of cooperative processes in the evolution of life and to the fact that the evolution of cells could not be fully understood as a simple progressive, incremental process but involved singularities with crucial macroevolutionary impact.

## 2.11 Formation of Modern Cell Biology and Methodical Trends in Twenty-First Century Cell Biology

Whereas nineteenth century biology had to decide which of the big theories were correct, late twentieth century cell biology was marked by the trend to put together the discoveries of genetics, molecular biology, biochemistry, and cytology into a congruent whole. Top-down (more and more detailed observation of tissue ultrastructure) and bottom-up (examining the properties of smallest functional components in the form of molecules and their relationships) approaches were eventually used together as a common tool set of a unified scientific field. Many processes were attributed to specific genes and their protein products. Proteins were successfully mapped into biochemical, signaling, and gene regulatory pathways. With the help of cell fractionation techniques and EM, combined with antibody staining, it became possible to map biochemical pathways and protein activities to specific subcellular compartments (Schuldiner and Schwappach 2013). The ability to maintain, grow, and manipulate cells outside organisms (a relatively simple task for plant cells), together with the expansion of live cell imaging techniques, especially discovery of genetically encoded fluorescent proteins, led to countless observations of dynamic processes in living cells. Cells have always been perceived as dynamic entities, but the new techniques allowed observation of molecular processes in vivo with the proper spatial and temporal context.

Emphasis has gradually shifted from the role of individual genes to how the actions of individual components within the cell collectively contribute to a particular process. This trend does not negate the earlier discoveries of twentieth century molecular biology in any sense, but demonstrates the importance of studying molecular components within live cells, taking into account structural and dynamic properties of the cellular environment. The cell has thus re-emerged as both a biological and an interpretational platform, connecting molecular mechanisms with macroscopic phenomena.

Several technological trends are typical for cell biology in this new millennium. First, improved techniques now allow cellular components and processes to be followed with greater and greater precision. The resolution of fluorescence microscopes is increasing in time and space, beyond the limitation imposed by the diffraction barrier (Wollman et al. 2015). The classical resolution limit of light microscopy has been surmounted by combination of fluorescence technologies and specialized fluorophore excitation methods. These techniques, along with sophisticated computer analyses, allow almost angstrom (Å) resolution in specific cases (Zeng and Xi 2016). Structural analyses of large macromolecular machines such as the ribosome (Yusupova and Yusupov 2017) and nuclear pore (Beck and Hurt 2017) are not uncommon. Fast tools for intracellular manipulation, such as optical tweezers (Ritchie and Woodside 2015), optogenetically activated proteins (Toettcher et al. 2011), and small photoactivated molecules (Hoglinger et al. 2014), now supplement traditional genetic and pharmacological tools.

Some of the new techniques are helping to bridge traditional approaches. For example, correlative light and electron microscopy enables live cell imaging. High resolution EM data can be acquired for a specific part of the cell after rapid freezing of the sample at a chosen time point (Kobayashi et al. 2016). During imaging mass spectrometry, specific regions of a cell/tissue are separately analyzed by mass spectrometry, which is thus enriched with spatial information (Asano et al. 2016). Analyses of protein structural properties, previously obtained by in vitro measurements, can be performed within the cellular environment in some cases (Schwamborn et al. 2016). Another dominant trend of contemporary cell biology is increasing experimental throughput with the help of automatized data acquisition and processing. Such tendencies were largely introduced for sequencing of whole genomes and transcriptomes but "omics" approaches are becoming widespread in connection with most techniques, including fluorescence microscopy (Mattiazzi Usaj et al. 2016), cell sorting (Warkiani et al. 2015), electron microscopy (Eberle et al. 2015), and structural biology (Grabowski et al. 2016).

## 2.12 Modular Cell Biology

It has become evident that, although some simple cellular functions are executed by a single molecular component (potassium transport through the plasma membrane via a membrane channel, metabolite conversion by a specialized enzyme), most cellular functions (growth regulation, cell differentiation, chemotaxis) arise from the interactions of many components (Hartwell et al. 1999). After decades of characterizing individual cell components and trends for their total catalogization, focus is now shifting from identifying individual parts to understanding their relationships, spatiotemporal associations, and collective behavior. Systems biology approaches rely on combining high-throughput data generated by various omics and quantitative computational analyses to generate new integrated insights into how individual parts produce emergent phenomena. Precise definition and methodology of systems biology is not unified and often elusive (Simpson 2016), but the main emphasis is on deducing the properties of interaction networks governing cellular processes. Ongoing debate exists about the need to change perception and scientific language if we are to understand cellular functions.

The concept of "modular biology" (closely linked with the concept of synthetic biology) is based on the realization that omics approaches alone are unable to uncover and understand the "design" or "engineering" (in a functional sense) principles of living organisms (Hartwell et al. 1999). Yuri Lazebnik has called for a new formalized language that is better suited to comprehend modules in living systems (Lazebnik 2002). Inspired by Hartwell et al. (1999), he uses the putative example of an effort to understand the functions of a radio and repair it using the methodology of molecular biology: dissecting the functioning system into a pile of random smaller parts or describing the effects of their removal (as in classical developmental genetics). Such an approach would undoubtedly lead to

identification of a few components that are crucial for functioning, and replacement of which would repair the radio if those components had been damaged. However, this procedure is futile if the individual components are functional but not tuned properly. Similarly, the quest of the pharmaceutical industry to find "miracle drugs" by identifying "critical molecular targets" does not often work because the malfunction may be the result of improper "tuning" of the whole system rather than damage to the critical molecular target.

On the other hand, the formal language of electronics (with components such as triggers and amplifiers) used by engineers provides direct insight into processes that the components are wired to perform. The analogy is not entirely fair because engineers have designed artifacts from first principles and formulated suitable language on the way, whereas the reverse-engineering approach of molecular biology meets systems that have evolved on their own for billions of years in complex environments. Nevertheless, biologists could learn more from taking an engineering perspective. Even the original models of gene expression regulation were inspired by Boolean logic, and many modern machines are now complex enough to foster further dialogue between biology and engineering, at least in the realm of signal transmission, processing, and interpretation (Csete and Doyle 2002). The concepts of amplification, adaptation (short and long term), robustness, insulation, attractors, bistability, waves and oscillations, memory switches, filtering, pattern recognition, discrimination of time series, hysteresis, complex logic gate operations, error correction, and coincidence detection should become staple parts of cell biology vocabulary. Cellular modules reflecting these concepts, rather than individual molecules, are of primary interest in understanding collective cell phenomena (Hartwell et al. 1999; Klipp and Liebermeister 2006; Lim et al. 2013; Mast et al. 2014). Novel bioinformatic methods can be used to search for similar network motifs, and it can be experimentally tested whether similar motifs play the same role in different contexts (Lim et al. 2013). The general functions of positive feedback (bistability, memory, switch-like behavior) and negative feedback (noise resistance, input-induced steady state) have been known for a long time (Lim et al. 2013).

The list of common motifs and architectures associated with specific functions in cells is now being expanded. For example, coherent feedforward loops often act as persistence detectors, which switch "on" only when the input persists for minimal amount of time (Lim et al. 2013). If the set of solutions for a particular problem is small enough, more analogies between artificial systems and cells should be possible to find and a table of frequent motifs with their functions established (Lim et al. 2013). There are even calls for verification of these rules by building minimal biological processing networks, with the use of a "synthetic biology" as the ultimate proof of understanding (Mast et al. 2014). However, it should be emphasized that networks and their motifs in living systems have their own specificities, because they often evolved to play multiple roles and work in unstable environments (Klipp and Liebermeister 2006). Yet, many modern artifacts are not dominated by minimal function but by modular buildup, which ensures robustness and further evolvability, so more similarities with evolving living systems could be

discovered in the future (Csete and Doyle 2002). The languages of modular cell biology and molecular cell biology are complementary, because the same functional motifs studied by modular biology can be implemented by many different molecular agents: "Cell biology is in transition from a science that was preoccupied with assigning functions to individual proteins or genes, to one that is now trying to cope with the complex sets of molecules that interact to form functional modules" (Hartwell et al. 1999).

## 2.13 Cells in Tissues: Molecular and Modular Mechanisms of Morphogenesis

Contemporary biology is again realizing the importance of an old wisdom that multicellular animals and plants are not composed of cells in a brick-like manner, but that tissues form specialized domains by cell growth, division, and differentiation. In addition to focusing on individual cell activity in this process, the dynamic integrated whole of the organism that produces and controls cells should be considered. As in cell biology, attempts have been made to understand multicellular developing systems in terms of the information processing networks of signaling pathways and gene expression regulation (Davidson 2010). It is also understood that, along with regulatory modules embodied in protein–protein interactions and gene promoter structures, the dynamic shape of tissue needs to be taken in account. For example, gradients of signaling molecules are dynamically reshaped by changes in tissue shape (Bollenbach and Heisenberg 2015). Therefore, each specific type of cell within an organism can be fully understood only within the context of its specific position within a tissue and its function. Bottom-up molecular and modular approaches must be complemented by top-down concepts that take into account the structure of developing tissues (Levin 2012).

Understanding both the modular and interconnected nature of living systems has allowed revival of the supracellular concept of field in developmental biology and its re-formulation in a framework compatible with molecular biology (Gilbert et al. 1996; Levin 2012). Such modular fields, displaying both autonomy and hierarchy and interacting with each other, have been proposed as mediators between genotype and phenotype in both ontogeny and evolution. Unlike some early field concepts, these fields are based on genetically defined interactions between cells. Their hierarchy and establishment are influenced by genetic information, but the field concept allows a shift of focus to the supracellular level of organization (Gilbert et al. 1996).

For a long time, the ECM was considered a passive material that filled the space between cells (Rozario and DeSimone 2010). Now it is understood as a dynamic repository of signaling molecules. The ECM can inhibit or facilitate signal spreading (Yan and Lin 2009; Rozario and DeSimone 2010), as well as store the morphogens and release them upon proteolytic degradation or stimulation by

additional signals (Rozario and DeSimone 2010). Moving cells reorganize the structure and position of ECM and ECM tracks the drive direction of cell migration (Rozario and DeSimone 2010). The actions of cells and ECM are thus bidirectional and complementary. More than a century after Roux defined a program of developmental mechanics, mechanical concepts are becoming the hallmark of mainstream developmental biology.

A program ridiculed by early developmental geneticists for not having achieved any mechanical understanding (Gilbert et al. 1996) now works fully within the framework of molecular biology. Developmental biology can also focus on mechanical aspects of development as a result of technological advances such as optical tweezers (Le et al. 2016), laser ablation of selected cells within tissue (Polacheck and Chen 2016), and atomic force microscopy to measure quantitatively the mechanical properties of cell/ECM surfaces at microscale resolution (Alcaraz et al. 2017). An increasing number of studies have demonstrated how the mechanical signaling within interconnected cellular–ECM nets strongly regulates growth, gene expression, and differentiation (Heisenberg and Bellaïche 2013), including mechanical aspects of regulation of cellular invasivity in normal development and in cancer establishment (Parekh and Weaver 2016).

## 2.14 Insights into Cytoplasm Structure in the Twenty-First Century

Together with the established tradition of associating cellular processes with membrane-bound organelles, attempts to comprehend the structure and properties of cytoplasm have reemerged 100 years after the decline of protoplasmic concepts, as nicely expressed in a quotation by T. Mitchison (2010): "Nothing epitomizes the mystery of life more than the spatial organization and dynamics of the cytoplasm."

The aqueous phase of the cytoplasm is not a bag of freely diffusing enzymes, as often wrongly perceived in the light of classical biochemistry, but is crowded with macromolecules. Diffusive transport and partitioning of macromolecules and organelles in cytoplasm is highly restricted by steric hindrance and by unexpected binding interactions (Luby-Phelps 2013). High viscosity and crowding are thought to play major roles in the mobility of cytoplasmic components. Mobility measurements by modern techniques indeed show behavior different from mere passive diffusion. Oddly, small proteins often move faster than inert molecules (Ross 2016). Weak interactions with surrounding cytoplasmic components possibly enhance their mobility. Recent advances have accumulated sufficient evidence for the existence of membraneless or "naked" compartments in the cytoplasm. Such compartments are formed by multivalent weak interactions between low complexity repeat domains and/or distorted hydrophobic domains (Luby-Phelps 2013; Uversky 2017). Self-interaction of domains ensures phase separation of the components from the rest of the cytoplasm. Upon formation of such a compartment by

polyvalent interacting proteins, monovalent interacting partners can enter the compartment and concentrate there.

Membraneless droplets could play a role in concentrating components of a cellular pathway without the need for a membrane barrier or other cage. Individual droplets of the same kind can split and coalesce, and components are constantly exchanged with the soluble pool (Weber and Brangwynne 2012). These structures thus possess a high level of internal dynamics and are characterized by liquid-like behavior, such as dripping, fusion, wetting, and the ability to become reversibly deformed when encountering a physical barrier (Uversky 2017). Droplets of different kinds (each based on a different self-interaction domain) can coexist within the cytoplasm without mixing together. Many such compartments are ribonucleoprotein granules consisting of long multivalent RNA molecules and specific RNA-binding proteins (Weber and Brangwynne 2012). Formation of membraneless compartments is condition-dependent, reversible, and controlled, including by posttranslational modification (Uversky 2017). The environment of these compartments is even more crowded than the rest of the cytoplasm (Uversky 2017). The combination of phase separation and molecular crowding can even trap together proteins with extremely low copy number (Wolde and Mugler 2014). The effects of crowding on the dynamics of signaling pathways, gene regulation networks, and metabolic networks are still not well understood, but crowding alters the diffusion of proteins and the kinetics of biochemical reactions (due to entropic changes), often in nonlinear dependence on the concentrations of molecules involved (Wolde and Mugler 2014).

Some of the ideas involving aqueous phase separation as a self-organizing mechanism trace back to 1899 or possibly earlier. E.B. Wilson proposed at the end of nineteenth century that non-membrane-bound compartments such as P-granules and Cajal bodies could be explained by the principles of colloid chemistry (Luby-Phelps 2013). Membraneless protein bodies of crystalline or quasicrystalline organization, probably formed by self-assembly, have also been known for some time. The shells of such compartments are permeable for small metabolites but otherwise keep the inside isolated from the rest of the cytoplasm (O'Connell et al. 2012). Most of these structures were discovered in bacterial cells, but examples from eukaryotes have also been described. In addition to the well-known polymerization of actin and tubulin into cytoskeletal fibers, some metabolic enzymes such as CTP synthase also tend to form fibers. Large-scale fluorescence microscopy screens revealed the localization of many supposedly cytoplasmic yeast proteins in fibers. The studies avoided overexpression artifacts and were supported by additional methods such as mass spectrometry for selected candidates (O'Connell et al. 2012). Packing of many proteins into as-yet uncharacterized structures is thus becoming evident.

Various roles for protein fibers and foci have been proposed, including efficient allosteric regulation, shielding of metabolic intermediates and their channeling into complex pathways, and storage of inactive proteins. Each of these functions has been demonstrated in particular cases but, for most proteins, the impact of assembly into aggregates is not known and the impact of the highly organized structure of the

cytoplasm is currently not well documented or understood. However, it is clear that certain emergent physicochemical properties of the cell interior cannot be revealed by reductionist experiments with a few isolated components. A challenge for postreductionist biochemistry is to study biochemical phenomena far from chemical equilibrium and under physiologically relevant conditions (i.e., inside cells, in complex cell extracts, or in crowded solutions) (Kyne and Crowley 2016).

## 2.15 Lipid and Membrane Research in the Twenty-First Century

Although support for the widespread existence of membraneless compartments in cells is accumulating, modern research also demonstrates the vital role of biological membranes. In interplay with cytoplasmic components, membranes expand the mechanisms of cell compartmentalization and functional regulation with additional layers of complexity. Lipids, although previously overlooked as mere passive components of membranes, are now appreciated as crucial determinants of membrane properties at different scales and are a key research topic in modern cell biology (Mouritsen and Bagatolli 2015). Improved lipidomic analyses demonstrate that the diversity of lipids could match the diversity of protein species in a eukaryotic cell and that the catalogue of lipid diversity is still expanding (Saliba et al. 2015). One year after the formulation of the fluid mosaic model of plasma membranes, it was hypothesized that more stable domains exist within evenly mixed membranes (Sezgin et al. 2017). This "lipid-raft" hypothesis, based on biochemical extractions indicating stable sphingolipid and sterol-enriched compartments within membranes, was never fully accepted. However, the expanded computational, biophysical, and biochemical tool set, including molecular dynamic simulations and advanced spectroscopic methods (Sezgin and Schwille 2011, 2012; Gumí-Audenis et al. 2016; Sommer 2013), is leading to better understanding of membrane heterogeneity at different spatial and temporal levels. Like macromolecules in cytoplasm, membrane components show anomalous diffusion and undergo clustering (Honigmann and Pralle 2016). Transient self-organized domains driven by segregation of components are reported at scales from a few molecules to micrometers. Moreover, the cortical actin cytoskeleton obviously fine tunes the organization of microdomains, not only by acting as a boundary to membrane protein diffusion but also by influencing lipid organization and phase transition, which can be further facilitated or suppressed by actin (depending on other specific conditions) (Honigmann and Pralle 2016). The existence of a fine actin–spectrin network has been observed in red blood cells and recently demonstrated in neurons with the help of super-resolution microscopy (D'Este et al. 2016), indicating a general cellular phenomenon. Fast local rearrangements of the domains as a result of feedback between the local phosphoinositide composition and actin cytoskeleton are also possible (Honigmann and Pralle 2016). Like the cytoplasmic cortex, the

ECM is believed to influence the mobility of membrane proteins, which has been demonstrated in the case of selective limiting of the mobility of plant plasma membrane proteins by the cell wall (Martinière et al. 2012). Differences in local lipid composition regulate the function of membrane proteins, and a substantial fraction of membrane lipids are bound to transmembrane proteins in the form of a hydrophobic solvation shell instead of being freely mobile within the bilayer (Poveda et al. 2017). The effects of lipid composition on the physical properties of a membrane are complex and difficult to predict. For example, cholesterol can increase or decrease local membrane fluidity depending on the other components (Schmid 2017).

Computational and experimental tools now allow assessment of the effect of specific compositions on membrane physical properties and protein structure in different situations (Poveda et al. 2017). Once cytoplasmic proteins are recruited to the membrane, the dimensionality of their mobility is reduced from three to two dimensions, increasing their effective concentration by orders of magnitude. Membranes thus serve as interaction platforms for proteins, which can be further fine-tuned by segregating interaction partners to specific microdomains (Honigmann and Pralle 2016; Stoeger et al. 2016). Membranes are now also understood to serve as tunable capacitors for integration and storage of information in the form of accumulation of specific signaling phospholipid species (Stoeger et al. 2016). Coincidence detection of more lipid species, or a specific lipid together with a protein interaction partner, regulates protein binding to the microdomains and membranes of different organelles (Saliba et al. 2015). Large-scale protein–protein interaction maps are now being complemented by high-throughput screens testing protein–membrane interactions and their dependence on the complex composition of the membrane and biophysical properties such as curvature (Saliba et al. 2015). The dynamic effects of lipid composition on cellular processes have been difficult to study, because membrane composition is subject to tight and fast regulation in the form of phospholipid headgroup modification, fatty acyl chain transfer, and movement of lipids between membrane leaflets (Sekereš et al. 2015). Furthermore, lipid transfer proteins in connection with membrane contact sites are being studied as regulated highways for lipid transport. Such a transport mechanism is possibly much faster than vesicular transport, previously considered to be the major agent of lipid movement between compartments (Jain and Holthuis 2017). Emerging technologies such as optogenetic activation of lipid-modifying enzymes (Idevall-Hagren and De Camilli 2015) and photoactivation of caged phospholipids (Hoglinger et al. 2014) now enable monitoring the effect of membrane composition changes on cellular processes at the physiological spatiotemporal scale.

## 2.16 Into the Unknown: The Future of Cell Biology

In addition to the increasing resolution and coverage of molecular measurements, discovery of some previously unknown fundamental components and mechanisms has been achieved. Discovery of RNA interference in the 1990s reshaped the perception of gene expression regulation and fostered growing interest in noncoding RNA species (Deniz and Erman 2016). There are also factors that probably have a large impact but are difficult to measure and factors whose existence we do not even suspect, the true "dark matter of cell biology" (Ross 2016). Examples of the former are the properties of intrinsically disordered proteins, small intracellular and intercellular DNA species, weak interactions impossible to detect using traditional biochemical methods, and intracellular distribution of ion species. The latter factors could be undiscovered protein–protein interaction motifs, exotic phases, undetected types of small molecules existing at low copy numbers, unknown posttranslational modifications, or new modes of collective behavior of biomolecules. With further improvement of available tools, it is possible that previously abandoned and possibly forgotten concepts in the framework of molecular biology will be revived, as happened with endosymbiotic theories and epigenetics. Cell biologists will continue to use the combination of top-down and bottom-up approaches. Detailed mechanistic characterization of individual components will be combined with large-scale systems level approaches, enabling identification of novel functional cellular modules. The future of cell biology (and of biology as a whole) also lies in capturing life processes simultaneously at different spatiotemporal scales and the integration of results into multiscale models, so that the relationship between the interactions of individual components and collective emergent phenomena can be understood.

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# Chapter 3
# Symbiotic Origin of Eukaryotic Nucleus: From Cell Body to Neo-Energide


**František Baluška and Sherrie Lyons**

Motto: *Omnis Energide e Energide*

**Abstract** Several aspects of the eukaryotic cell suggest that the nucleus is of symbiotic origin. The nucleus forms, via its perinuclear structures, the primary eukaryotic agent known also as the "cell body" or "energide." New energides are generated only from other energides, as is the case for all other endosymbiotic organelles. Moreover, the energide can use its secretory apparatus to generate de novo the cell periphery apparatus. In contrast, the energide cannot be generated de novo. All this suggests that the energide was the primary symbiont of the eukaryotic cell and enslaved the host cell by stripping it of its DNA. The energide took control over the host cell that provided it with a protective niche. This feature, supported by other relevant data, suggests that the endoplasmic reticulum (ER) is a secondary organelle generated by the outer portion of the nuclear membrane. The ER represents a specialized domain of the outer nuclear envelope, which orchestrates the energide's secretory and lytic activities via the ER network, Golgi apparatus, autophagy network, and lysosomes. In this way, the energide integrates the eukaryotic cell via ER/organelle/plasma membrane contact sites into a coherent agent of eukaryotic life. In addition, the plasma membrane provides feedback to the energide and renders protection via the plasma membrane-derived endosomal network. Recent new discoveries suggest archaeal origins for both the energide and its host cell.

Dedicated to the memory of Peter W. Barlow (1942–2017).

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## 3.1 Introduction

All life on Earth is cellular life. The most important event in the evolution of multicellular organisms was undoubtedly evolution of the nucleus and the complex cellular organization of the eukaryotic cell. However, the evolutionary origin of the nucleus, although very ancient, remains shrouded in mystery. The various efforts to illuminate how the nucleus formed remain unconvincing and enmeshed in controversy. The most contentious issue is whether the nucleus emerged, as most of the current popular theories suggest, via the autogenic mode (Taylor 1976; Wilson and Dawson 2011; Baum 2015) or whether the nucleus is a vestige of ancient endosymbiotic events (Lake and Rivera 1994; Gupta and Golding 1996; Horiike et al. 2001; Hartman and Fedorov 2002; Kutschera and Niklas 2005). Early proposals on the endosymbiotic origin of the nucleus go back to Wilhelm Pfeffer, Theodor Boveri, and Konstantin Mereschkovsky as discussed in Wilson (1925), Lake and Rivera (1994), and Sapp et al. (2002). The nucleus could result from either ancient cellular parasitism or predation (Cavalier-Smith 2002; Davidov and Jurkevitch 2009; de Nooijer et al. 2009). These two very different ideas are difficult to reconcile and it is almost impossible to prove either of them conclusively due to the absence of surviving intermediary stages or any convincing fossil traces. The recently discovered eukaryotic fossils of ancient multicellular eukaryotes show large nucleus-like organelles in the well-preserved cells (Bengtson et al. 2017). This discovery reveals that the eukaryotic nucleus was already present 1.6 billion years ago, suggesting that emergence of the eukaryotic nucleus was a relatively fast process, not compatible with slow autogenic scenarios (Wilson and Dawson 2011; Baum 2015; López-García and Moreira 2015). Nevertheless, the evolutionary origin of the eukaryotic nucleus remains obscure (López-García and Moreira 2015). As remarked by Lynn Margulis and coworkers, any decent hypothesis on the origins of the nucleus and eukaryotic cell must account for the common evolutionary origins and intimate relationships between eukaryotic nuclei, centrioles, centrosomes, basal bodies, microtubules (MTs), and MT-based eukaryotic flagella (Chapman et al. 2000; Margulis et al. 2000; Dolan et al. 2002). This is a strong argument against slow autogenic scenarios.

Why has it been so difficult to elucidate the true evolutionary nature of the eukaryotic cell endowed with a nucleus? The reason is that cell theory maintains that cells can only arise from preexisting cells and that the cell is the smallest independent unit of life. Most of the numerous theories proposed so far have been based on diverse autogenic scenarios of eukaryotic cell evolution (Wilson and Dawson 2011; Baum 2015; Devos et al. 2014). In 1910, Konstantin Mereschkowsky proposed a theory of symbiogenesis, arguing that complex large cells evolved from a symbiotic relationship between smaller cells, but this bold theory was essentially ignored for half a century. Beginning in the 1960s, Lynn Margulis popularized and further developed Mereschkovsky's ideas, but she was also marginalized for many years. She proposed that organelles such as the chloroplast and mitochondria were once free living cells. Her idea gained more credibility with the discovery that such

organelles contained DNA that was distinct from nuclear DNA, but it was not until the advent of detailed DNA sequencing that her ideas were fully accepted. Lynn Margulis's (at that time Lynn Sagan) landmark paper from 1967 (Sagan 1967; Taylor 1974; Goldman 2012; Martin 2017) was rejected for publication by 15 journals (Lake 2011; Goldman 2012; Gray 2017) before it was accepted by the *Journal of Theoretical Biology* (Sagan 1967). The revolutionary message of this paper was that eukaryotic cells are multigenomic cells (or cells within a cell) generated by endosymbiosis (Sagan 1967) and are not autogenously generated via standard microevolutionary processes such as point mutations and duplications of genes and genomes (Martin 2017). Recently, the *Journal of Theoretical Biology* celebrated this milestone paper with a series of papers highlighting the importance of her article (Lane 2017; Lazcano and Peretó 2017; Martin 2017). We suggest that without the discovery of bacterial genes in mitochondria and plastids, the endosymbiotic origin of these organelles would probably still not be accepted because the dominating concept strongly prefers, as long as no obvious organelle genome is present, the autogenous generation of eukaryotic organelles (De Duve 2007). We suggest that this preference for autogenous generation of the eukaryotic nucleus is the reason why the "cell body" concept (Mazia 1993; Baluška et al. 1997, 2004a, b) has not been widely adopted, in spite of a great deal of evidence that supports it.

## 3.2 The Dual Nature of the Eukaryotic Cell: Cell Periphery Complex Versus Energide

What is relevant to our understanding of the evolutionary origin of the eukaryotic cell is the dual nature of most of its constituents and processes. The dual nature of the eukaryotic cell is seen in vesicle trafficking (exocytosis and endocytosis), the cytoskeleton (actin-based and tubulin-based), and in the cytoarchitecture (the nucleus with its perinuclear apparatus and the cell periphery complex). Duality is a characteristic feature of endomembrane/vesicle systems such as the COPI/COPII vesicle coat complexes of the nuclear envelope (NE); the endoplasmic reticulum (ER) and Golgi apparatus (GA); and the clathrin coat complexes of the plasma membrane (PM), endosomes, and trans-Golgi networks (Holstein 2002; Bonifacino and Lippincott-Schwartz 2003; Robinson 2015; Dergai et al. 2016; Rout and Field 2017). Clathrin and COPs represent ancient vesicle-generating complexes, subunits of which assemble cage-like scaffolds around nascent vesicles to drive eukaryotic vesicle formation (Rout and Field 2017).

The nucleus is primarily associated with the microtubular cytoskeleton and its perinuclear microtubular organizing centres (Baluška and Barlow 1993; Baluška et al. 1997, 2004a, b), whereas the actin cytoskeleton supports endocytic networks at the cell periphery complex (Šamaj et al. 2005; Scita and Di Fiore 2010; Sigismund et al. 2012). We have proposed, in our extension of Daniel Mazia's

cell body concept, that a hypothetical tubulin-based guest cell invaded the hypothetical actin-based host cell, eventually becoming a nucleus with associated structures (cell body) in the host cell (Baluška et al. 2004a, b). Later, we realized that the original "energide" concept of Julius Sachs preceded Daniel Mazia's cell body concept by more than 100 years (Sachs 1892a, b; Mazia 1993; Baluška et al. 2006a). However, whereas Sach's energide suggestion was largely hypothetical, advances in microscopy and biochemical analysis meant that Mazia could cite concrete evidence to support his cell body claims (Mazia 1984, 1987, 1993). We have reformulated these concepts into an updated neo-energide concept. For the early version of the neo-energide concept, see Baluška et al. (2006a); Nicholson (2010) and Lyons (2018) give a more general discussion on these interesting historical aspects.

## 3.3 Nucleus-Based Cell Body/Energide as the Primary Unit of the Eukaryotic Cell

The nucleus with its microtubule–endoplasmic reticulum–Golgi apparatus (MT-ER-GA) complex acts as the primary agent of the eukaryotic cell. It controls and manipulates the cytoplasm and the cell periphery apparatus in a manner resembling niche generation and maintenance. That the primary nature of the nucleus includes its perinuclear cytoskeleton/membrane assemblies follows from the sequence of events during eukaryotic cell division, when division of the nucleus (mitosis) invariably precedes cell division (cytokinesis). This primary nature of the nucleus contradicts the many autogenic theories that posit autogenic scenarios for the evolutionary origin of the eukaryotic nucleus. Importantly, the cell periphery apparatus with all the cytoplasm cannot generate a new nucleus if removed from the cell experimentally. On the other hand, the nucleus with associated cytoplasm can generate a new cell periphery apparatus, as seen in plant cytokinesis, cellularization of syncytial tissues, and wounded siphonous algae (Baluška et al. 2004a, b, 2006a, b).

The nucleus is invariably enclosed, as are all symbiotic organelles, with a double membrane. The outer membrane of the NE is continuous with the ER membranes, which spin off endomembranes that make up the secretory system of the eukaryotic cell (including the GA). Both the ER and GA are integrated into a functional unit via cell body perinuclear microtubules (MTs) and F-actin (Baluška et al. 1997, 2004a, b). MTs are primarily seeded either at the perinuclear centrosomes/centrioles of animal cells (Rieder et al. 2001; Vertii et al. 2016) or at the whole nuclear surface, which acts as the primary microtubule organizing center (MTOC) of plant cells (Mizuno 1993; Stoppin et al. 1994, Baluška et al. 1997, 2004a, b, 2006a; Shimamura et al. 2004; Nakayama et al. 2008). MTs are also found in large ceonocytic muscle cells known as myotubes (Kronebusch and Singer 1987; Tassin et al. 1985; Folker and Baylies 2013).

Daniel Mazia elaborated on the original ideas of Theodor Boveri (Scheer 2017), who wrote that the centrosome was the "autonomous permanent organ of the cell. . . the dynamic center of the cell. . . the true division-organ of the cell. . . coordinating nuclear and cytoplasmic division" (Mazia 1984). For Mazia, centrosomes were potentially far more than just the organizer and initiator of MT polymerization (Mazia 1984, 1987, 1993). They were "bearers of information about cell morphology." For cells of higher plants, lacking corpuscular centrosomes and centrioles, Mazia proposed the concept of flexible centrosomes (Mazia 1987). This concept has gained significant support in the last three decades (Mizuno 1993; Stoppin et al. 1994; Baluška et al. 1997, 1998, 2012; Binarová et al. 2000; Schmit 2002; Fant et al. 2009; Srsen et al. 2009; Petrovská et al. 2015; Chen et al. 2017; Yamada and Goshima 2017). As proposed by Mazia (1984, 1987), both chromosomal and centrosomal cycles are closely integrated during the cell cycle (Baluška et al. 1997).

### *3.3.1 Omnis Energide e Energide*

Although the current dominant view remains the autogenic origin of the eukaryotic nucleus, many findings in cell biology directly conflict with this hypothesis. First, the nucleus cannot be assembled de novo; it can only be generated from another nucleus. Second, cell division is preceded by nuclear division. The famous dictum by Rudolf Virchow, *Omnis Cellula e Cellula* (cells come only from cells), was modified by Walther Flemming into *Omnis Nucleus e Nucleo* in 1882 (Flemming 1882; Osorio and Gomes 2013), but should be re-formulated into a new dictum *Omnis Energide e Energide* (energides come only from energides). In fact, Virchow's dictum from 1855 was first proposed by François-Vincent Raspail in 1825 (Tan and Brown 2006) as a rejection of the concept of spontaneous generation, which postulated that living organisms can be spontaneously generated from nonliving matter. We have revived Daniel Mazia's concept of the cell body, which was virtually identical to Julius Sachs concept of the energide (Sachs 1892a, b; Baluška et al. 2006a).

The cell body/energide concept postulates that it is not the cell itself but the nucleus with some associated structures that represents the basic, primary, and fundamental unit of eukaryotic organisms (Baluška et al. 1997, 1998, 2001, 2004a, b, 2006a, b, 2012). We argue that the nucleus is a vestige of the first primary endosymbiont and keeps its autonomy and primacy in the eukaryotic cell. In the energide concept, the cytoplasm and the cell periphery complex (PM with its endocytic recycling apparatus and cell wall/extracellular matrix) are vestiges of the host cell, whose activities are now tightly controlled by the nucleus/cell body/energide via its MT cytoskeleton and ER-GA networks. When formulating his original energide concept, Julius Sachs was not able to observe the MT cytoskeleton, although it plays a central role in the cell body/energide activities (Sachs 1892a, b), as proposed by Daniel Mazia in 1993 and in our series of conceptual

papers published between 1997 and 2012 (Baluška et al. 1997, 1998, 2001, 2004a, b, 2006a, b, 2012).

The primary nature of the energide over the eukaryotic cell is demonstrated by the ability of postmitotic energides to generate new cells using its endosomes (storing cell wall/extracellular matrix enclosed by PM), which were generated via premitotic energides during the preceding interphase (Baluška et al. 2002, 2005, 2006a, b; Dhonukshe et al. 2006, 2007; for animal cells see Ai and Skop 2009; Elia et al. 2011; Schiel et al. 2013; Bhutta et al. 2014; Gulluni et al. 2017). Another indication of the primary nature of the energide over the eukaryotic cell is that cell division invariably starts with energide division. Cell division (the cell periphery and cytoplasm) or cytokinesis is accomplished only after energide division is completed. Cytokinesis can be incomplete (leaving out cell–cell channels) or even fully absent, resulting in syncytia (giant cells with many nuclei) (Baluška et al. 2004a, b, 2006a, b). Several examples are relevant: Active energides generate new cells during developmentally controlled cellularization processes, such as occurs in the syncytial blastoderm during insect embryo development (Sokac and Wieschaus 2008a, b; Rikhy et al. 2015; Sherlekar and Rikhy 2016) and in the syncytial endosperm during embryogenesis of flowering plants (Olsen 2001). A more striking example, but lesser known, is regeneration of cells from nuclei released from wounded syncytial algae within small protoplasts (Kobayashi and Kanaizuka 1977; O'Neil and La Claire 1984; Pak et al. 1991; Kim et al. 2001, 2002).

## 3.4 The Dual Nature of the Eukaryotic Cell: Energides Build Their Niches

From the perspective of the energide, the host cell represents a niche for the energide. The cell periphery complex, equipped with an extracellular matrix or cell wall, provides an effective shelter while the PM has receptors for abiotic and biotic parameters to feed the energide with sensory information about the environment and/or its tissue-specific context. Using DNA and the cytoplasmic cytoskeleton with its associated ER-based endomembrane system, the energide can control the composition not only of the cytoplasm in which it is embedded, but also of the PM and surrounding extracellular matrix, generating together the sheltering cell periphery complex.

The cell periphery complex is organized via the PM activities connected to the cytoplasmic cytoskeleton, especially the actin cytoskeleton. In addition to receiving exocytic secretory vesicles from the anterograde membrane flow initiated at the outer nuclear membrane, the PM organizes its own membraneous apparatus via endocytosis, which generates the retrograde membrane flow initiated at the PM (Sigismund et al. 2012). These two membrane flows are based on different coat complexes: the COPI/COPII complexes of the cell body/energide and the clathrin coats of the PM-organized host cell membranes (Bonifacino and

Lippincott-Schwartz 2003; Rout and Field 2017). The endocytic networks, organized by the PM, provide the energide with all relevant sensory information from the outside extracellular space (Šamaj et al. 2005; Polo and Di Fiore 2006; Sorkin and von Zastrow 2009; Barbieri et al. 2016).

### 3.4.1 *Nuclear Pore Complexes as Prototypic Cell–Cell Channels to Control the Energide's Niche*

To establish cytoplasmic access to their gene expression products, energides maintain cell–cell channels, known as nuclear pores, which are embedded within the peripheral part of the nuclear skeleton, which is composed of lamins and lamin-like proteins. These proteins are similar to the intermediate filaments and have an ancient origin (Kollmar 2015; Koreny and Field 2016). That the nuclear cytoskeleton is composed of a unique class of cytoskeletal proteins is very strong support for the endosymbiotic origin of energides. Moreover, this is also supported by the close structural and functional similarities between nuclear pores and the cell–cell channels of plant cells (known as plasmodesmata) and fungal septal pores (Lucas et al. 1993; Lucas and Lee 2004; Lee et al. 2000; Baluška et al. 2006a; Bloemendal and Kück 2013). Significantly, there are also close connections between the nuclear pore complexes and centrosomes and between the nuclear pore complexes and ciliary gating zones (discussed in Sect. 3.4.1). With respect to the evolutionary origin of the nuclear pore complexes, these supercomplexes contain at their core scaffold proteins similar to the vesicle coat complexes COPI and COPII (Field et al. 2014; Rout and Field 2017). In addition, the chimeric nature of nuclear pore complexes, based on both COPI- and COPII-like systems, closely resemble the flagellar entry domain, which is also a chimera of COPI- and COPII-like systems (Rout and Field 2017). This similarity between the flagellar entry domains and the nuclear pores strongly suggests that these structures evolved together.

### 3.4.2 *Nuclear Pores Are Embedded Within the Ancient Lamina-Based Nucleoskeleton*

The separate evolutionary origin of the nucleus is supported by its unique nucleoskeleton that contains evolutionarily ancient lamins and has no similarities to the tubulin and actin-based cytoskeleton. Nuclear pores are embedded within skeletal meshworks assembled from the lamin and lamin-like proteins that underlie the inner part of the NE and also control chromatin complex organization throughout the nuclear interior (Simon and Wilson 2011; Kind and van Steensel 2014; Harr et al. 2015; Gesson et al. 2016; van Steensel and Belmont 2017). This lamin-based nucleoskeleton might also interact with the still-elusive nuclear matrix that is

associated with the RNAs in the ribonucleoprotein networks that control gene expression via chromatin structures (Smetana et al. 1963; Pederson 2000; Nickerson 2001; Dobson et al. 2017). It has been proposed that the nuclear matrix lamin-based nucleoskeleton and the chromatin complex co-evolved during the early evolution of the eukaryotic cell (Wen and Li 1998; Peter and Stick 2015; Koreny and Field 2016) and that the cytoplasmic intermediate filaments evolved secondarily from the more ancient nuclear lamins (Peter and Stick 2015; Koreny and Field 2016). This strongly supports the ancient symbiotic origin of nuclear lamins and the eukaryotic nucleus. Interestingly, lamins and centrins connect centrioles/centrosomes with the nuclear periphery (Gräf et al. 2015).

## 3.5 The Dual Nature of the Eukaryotic Cell: Endomembranes and Vesicles Are Organized Via the Plasma Membrane and the Nuclear Envelope

The current dominating autogenous concept for the evolutionary origin of the eukaryotic nucleus states that the NE was generated via ER membranes enclosing a genetic apparatus based on DNA and RNA networks (Cavalier-Smith 1987, 1988; Wilson and Dawson 2011; Devos et al. 2014; González-Sánchez et al. 2015; Martin et al. 2015). However, this scenario has problems with the fact that the inner NE differs from the outer NE in its inherent association with lamins and the nucleoskeleton; whereas the outer NE gives rise to the ER membranes. Moreover, it also ignores the duality of the endomembrane/vesicle systems. The energide (guest cell) outer NE and ER-derived endomembrane vesiculation is based on the COPI and COPII coat complexes, whereas the PM-based (host cell) vesiculation is driven by the clathrin complexes. Furthermore, the PM is inherently linked with and organizes the extracellular matrix/cell wall molecules. In other words, the PM differs from the ER membranes so significantly that this precludes their common evolutionary origin. The criticism that symbiotic theories of nuclear origin are not compatible with the existence of nuclear pores (Cavalier-Smith 1987, 1988; Wilson and Dawson 2011; Devos et al. 2014) is now outdated because nuclear pores emerge as classical cell–cell channels, resembling plant plasmodesmata or fungal septal pores (see Sect. 3.4.1). Interestingly, the ancient protein centrin organizes not only centrioles and the nucleus–basal body contractile connectors in flagellated unicellular organisms such as *Chlamydomonas* (Salisbury et al. 1988; Wright et al. 1989; Taillon et al. 1992; Koblenz et al. 2003), but also connects centrioles/centrosomes to the nuclear periphery (Gräf et al. 2015). Centrin is a component of nuclear pores (Resendes et al. 2008) as well as of plant-specific cell–cell channels (plasmodesmata) (Blackman et al. 1999).

### 3.5.1 The Endoplasmic Reticulum as a Specialized Extension of the Outer Part of the Nuclear Envelope

In autogenous theories, the NE is considered to be an extension of ER membranes (Cavalier-Smith 2010; Hetzer 2010; Ungricht and Kutay 2017). By the same token, the ER could represents a specialized extension of the outer part of the NE. Importantly, the outer and inner membranes of the NE have different properties, proteins, and functions (Katta et al. 2014; Ungricht and Kutay 2015; Smoyer et al. 2016). Only the outer part of the NE is continuous with ER membranes, and the lumen of the NE has different properties from the lumen of the ER. Whereas the inner nuclear membrane associates with the nuclear skeleton, especially with its lamin-based meshworks (Hetzer 2010), the outer NE associates with the cytoplasmic cytoskeleton (Gerace et al. 2012; Tapley and Starr 2013; Navarro et al. 2016).

### 3.5.2 The Golgi Apparatus as Specialized Extension of the Endoplasmic Reticulum

In the energide view of the eukaryotic cell, the ER membrane is an extension of the outer nuclear membrane, the GA is an extension of ER membrane, and the trans-Golgi network (TGN) is derived from GA membranes. This means that the outer NE is a "mother" membrane of the energide (guest cell), whereas the host cell-based PM generates the endosomal vesicles and endosomal networks of eukaryotic cells (Šamaj et al. 2005; Polo and Di Fiore 2006; Sorkin and von Zastrow 2009; Barbieri et al. 2016). The ER and GA membranes are the main sites of lipid biosynthesis and are enriched with glycerophospholipids, but contain only small amounts of sphingolipids and the nonpolar structural lipids of cell membranes such as sterols. In contrast, the PM and endosomes contain many more structural sterols, often assembled in the form of "lipid rafts," which are important for signaling (Simons and Vaz 2004; Lingwood and Simons 2010; Simons and Sampaio 2011; Sezgin et al. 2017). As discussed above, the NE, ER, and GA membranes (energide/cell body endomembranes) rely on the COPI/COPII coat complexes to generate vesicles and other membraneous carriers, but the PM and endosomes (host cell endomembrane system) rely on the clathrin coat complexes (Bonifacino and Lippincott-Schwartz 2003; Rout and Field 2017).

## 3.6 The Dual Nature of the Eukaryotic Cell: Tubulin-Based Guest and Actin-Based Host

There are several models of the evolutionary origin of eukaryotes. Despite the acceptance that endosymbiosis played a role in the evolution of eukaryotic cells with regard to the acquisition of mitochondria and chloroplasts (Lake 2011; Gray 2017; Martin 2017), the origin of eukaryotes is still considered mainly under autogenic scenarios (Baum 2015). In our proposed scenario, a tubulin-based invader cell acts as a parasite that effectively strips the host cell of all its DNA molecules and completely eradicates its genome. This process not only masks the origin of the organismal/cellular host, but also might allow the invader guest cell to take control over the host cell and act as the nucleus of the contemporary eukaryotic cell. It is possible that the invader cell was a ciliated cell and eventually transformed the eukaryotic nucleus. After the loss of cilia from the guest cell, the centrosomes associated with radiating MTs retained close contacts with nuclear surfaces (Baluška et al. 1997, 2004a; Janota et al. 2017). Intriguingly, there are close similarities between the nuclear pore complex and the ciliary pore complex (Devos et al. 2004; Dishinger et al. 2010; Field et al. 2011; Onischenko and Weis 2011; Kee et al. 2012; Takao et al. 2014). A crucial finding is that molecules such as importins, nucleoporins, and Ran-GTP gradients that control gating of the ciliary entry zone also control gating of the nuclear pores (Kee et al. 2012; Kee and Verhey 2013; Takao et al. 2014, 2017; Takao and Verhey 2016; Torrado et al. 2016). In addition, several cilia-associated proteins have nuclear roles (McClure-Begley and Klymkowsky 2017). All of these discoveries strongly suggest that gating of both nuclear pores and ciliary pores have common evolutionary origins because they make use of the same molecules and similar mechanisms. These commonalities between nuclear pores and cilia pores fit nicely into the concept of a symbiotic origin of the eukaryotic nucleus (Lake and Rivera 1994; Margulis et al. 2000; Dolan et al. 2002).

The hypothetical ciliated/flagellated symbiotic guest cell lost all the cilia/flagella, which turned subsequently into nuclear pores during transformation of the primary endosymbiont into the eukaryotic nucleus. In addition, during this transformation into the eukaryotic nucleus, the symbiotic guest cell was effective in taking control over the endomembranes/vesicles of its host cell via stealing all the genome (currently cytoplasm and the cell periphery complex), which was left without any DNA. This allowed the guest cell to take complete control over the host cell and to transform into a full-blown cell body/energide. As the nucleus also accumulates DNA/genes from other endosymbiotic organelles, its chimeric status is not so surprising. The close similarities between the perinuclear centrioles/centrosomes and flagellar basal bodies (Azimzadeh 2014) also support this endosymbiotic scenario for the evolution of the cell body/energide. Interestingly, choanoflagellata and the early diverging protist *Giardia lamblia* (Elias et al. 2008) lack centrosomes and their basal bodies function as centrosomes during mitosis (Dawson 2010; Dawson and House 2010; Karpov 2016). This suggests that the evolution of

flagellar basal bodies preceded the evolution of centrioles/centrosomes (Bornens and Azimzadeh 2007).

This attractive scenario also gains support from *G. lamblia*. Some of its flagella are initiated at the nuclear surface and their very long axonemes have long cytoplasmic regions before they exit the cell as membrane-bound flagella (Dawson and House 2010; McInally and Dawson 2016; Hardin et al. 2017). There are several other features that support the idea that *G. lamblia* is close to the hypothetical MT-based guest cell that invaded the hypothetical actin-based host cell (Lake and Rivera 1994; Baluška et al. 1997, 2004a). *Giardia* has a reduced actin-based cytoskeleton, lacking actin-binding proteins and myosins (Hardin et al. 2017). Interestingly, the myosin-independent cytokinesis in *Giardia* is based on nucleus-associated flagella, which coordinate vesicle trafficking. All eight flagella are retained during *Giardia* mitosis and their basal bodies migrate to generate four spindle poles, acting as MTOCs of mitotic spindles (Dawson and House 2010; McInally and Dawson 2016). Flagella of *Giardia* are internalized only during encystation, but their rudiments still beat inside newly formed cysts (Midlej and Benchimol 2009). *Giardia* have only minimal sets of organelles and lack mitochondria, peroxisomes, classical GA, ER, and canonical lysosomes; they contain ER-like tubulovesicular compartments, which fulfill the roles of the GA (Zamponi et al. 2017; Touz and Zamponi 2017) and interact with clathrin-based vacuoles (Faso and Hehl 2011; Abodeely et al. 2009; Zumthor et al. 2016). Interestingly, the mitochondria-like mitosomes of *Giardia* are constitutively associated with ER membranes (Voleman et al. 2017).

Another relevant finding is that the nuclear pore complexes have regulatory roles in the insertion of spindle pole bodies into the NE during spindle pole assembly and duplication in budding yeast (Jaspersen and Ghosh 2012; Rüthnick et al. 2017). Moreover, there are structural similarities between spindle pole bodies and nuclear pore complexes inserted into the NE (see figures 1 and 3 in Jaspersen and Ghosh 2012). As these spindle pole bodies represent the centrosome counterparts found in other organisms, these findings suggest close evolutionary connections between the nuclear pores and centrosomes.

There is also strong evidence for the perinuclear origin of eukaryotic cilia. Syne proteins are involved in the docking and anchoring of nuclei to the neuromuscular synaptic junctions (Apel et al. 2000; Grady et al. 2005; Ruegg 2005; Zhang et al. 2007; Espigat-Georger et al. 2016). Recently, Syne proteins were also discovered to be integral components of ciliary rootlets (Potter et al. 2017). This finding, together with the structural and molecular similarities between the nuclear pore complex and the ciliary pore complex, suggests a common symbiotic origin of the nuclei, nuclear pores, and cilia/flagella of eukaryotic cells. Furthermore, the eukaryotic flagellar apparatus is an ancient organelle that is invariantly associated with basal bodies and acts, similarly to the nucleus-associated centrosomes, as a primary MTOC of eukaryotic cells (Yubuki and Leander 2013; Azimzadeh 2014; Gräf et al. 2015). Significantly, flagellar basal bodies of unicellular organisms such as the green alga *Chlamydomonas* and the protozoan *Giardia* are connected to, and anchored at, the nuclear peripheries via contractile centrin-based fibers (Salisbury 1988; Salisbury

et al. 1988; Wright et al. 1989; Taillon et al. 1992; Koblenz et al. 2003; Benchimol 2005, 2007; Dawson and House 2010). Close structural associations and functional integration of centrosomes and chromosomes provide very strong support for the cell body/energide concept (Baluška et al. 1997, 2004a, 2006a, 2012). In support of the symbiotic origin of the eukaryotic nucleus, synaptic proteins Homer and Flotillin were proposed to be localized in the nuclei of the last common ancestor of metazoans, as is the case for the choanoflagellate *Salpingoeca rosetta*, one of the closest living relatives of metazoans (Burkhardt et al. 2014; Burkhardt 2015).

## 3.7 Archaea and the Ancient Symbiotic Origin of Eukaryotes and Their Nuclei

The defining characteristic of eukaryotic cells is the presence of a nucleus bound by a double membrane. As in endosymbiotic plastids and mitochondria, the outer and inner membranes of the NE have different functions and a different molecular basis. According to the Schnepf theorem, which posits that biological membranes separate plasmatic from nonplasmatic phases (Bothe and Melkonian 2016; Moog and Maier 2017), this feature also suggests a symbiotic origin of the eukaryotic nucleus. Unicellular organisms with a nucleus have been defined as eukaryotes, and those without a nucleus are considered to be a prokaryote or bacteria. However, a third major group has now been proposed, the archaea. Superficially, archaea appear to be an unusual and very old group of bacteria. But, the advent of detailed molecular sequence data has shown that they seem to be no more closely related to bacteria than to eukaryotes. Instead of two kingdoms, Carl Woese suggested three kingdoms: Bacteria, archaea, and Eukarya (Woese 2004a, b). However, from the 1970s through the mid-1990s, the relationship of the three groups was problematic: Were archaea the oldest? Where did the eukaryotes come from? Were eukaryotes relatively modern, or did they arrive soon after life began, perhaps evolving from archaea? Were they the product of several fusion events from several different bacteria?

The symbiotic origin of chloroplasts and mitochondria had been postulated for some time, but by the 1990s, as a result of detailed sequence data, it was finally accepted that both organelles were once free living bacteria that had been engulfed by another organism. The biggest surprise concerned the origin of the nucleus. The nuclear genome is a molecular chimera with inputs from all three groups. Informational/translational genes came from archaea whereas metabolic genes came from bacteria. What is most significant for our purposes is that cytoskeletal genes appear to have come from an equally ancient eukaryotic cell type. Thus, the eukaryotic cell seems to be the product of at least two proto-eukaryotic cells, with input also from the other groups. Lateral gene transfer was so pervasive in the ancient world that it may be impossible to determine the exact relationship of these groups to each other (Woese 2002, 2004a). However, the importance of these findings suggests that we

need to rethink the concept of the eukaryotic cell. In Daniel Mazia's words "something truly fundamental is missing in our image of the cell" (Mazia 1987; Baluška et al. 2004a). The cell body/energide should be considered the smallest independent unit, rather than the cell. However, because the evolutionary origin of the nucleus (cell body/energide) remains obscure, our full understanding of the eukaryotic cell is limited.

Recent advances in our understanding of archaea provide strong evidence that the eukaryotic lineage evolved from within the archaea, and that Eukarya and archaea are intimately related (Guy and Ettema 2011; Guy et al. 2014; Williams et al. 2013; Spang et al. 2013, 2015; Williams and Embley 2014; Klinger et al. 2016; Surkont and Pereira-Leal 2016; Spang et al. 2017; Zaremba-Niedzwiedzka et al. 2017). However, a couple of different scenarios have also been postulated (Rochette et al. 2014; Spang et al. 2017). It seems that the elusive ancestral host cell, receiving endosymbiotic bacteria that eventually transformed into the mitochondria of eukaryotic cells, was a member of the archaeal cell lineage. Moreover, this archaeal host cell also accommodated another endosymbiotic (also archaeal) cell, which transformed into the nucleus. Our favored version of the endosymbiotic theory for the origin of the nucleus posits that the guest cell delivered a centrosome/centriole complex with MTs into the host cell. The identity of this guest cell is unknown but it might be another kind of archaeal cell, because archaea contain both histones and nucleosomes that generate a chromatin complex closely resembling the eukaryotic chromatin complex (Pereira and Reeve 1998; Bailey et al. 2002; Reeve et al. 2004; Ammar et al. 2012; Nalabothula et al. 2013; Mattiroli et al. 2017). Most importantly, eukaryotic histones share a common ancestry with archaeal histones (Reeve et al. 2004). Furthermore, archaeal DNA replication resembles the eukaryotic counterparts at both organizational and mechanistic levels (Samson and Bell 2016; Samson et al. 2016).

Another possibility is that an unknown ancient bacteria acted as the elusive guest cell. Discovery of bacterial tubulins assembling an eukaryotic-like microtubule cytoskeleton (Schlieper et al. 2005; Pilhofer et al. 2011; Deng et al. 2017; Díaz-Celis et al. 2017; Trépout and Wehenkel 2017) and interacting with bacterial kinesin (Akendengue et al. 2017; Deng et al. 2017) are supportive in this respect. However, other findings suggest instead that these proteins have eukaryotic origins and were obtained by bacteria via horizontal gene transfer (Schlieper et al. 2005; Martin-Galiano et al. 2011; Akendengue et al. 2017).

An alternative scenario postulates that this ancient guest cell was an unknown unicellular organism equipped with a flagella-like organelle (or exo-symbiont) based on the centrosome/centriole complex with MTs. Flagellated protists such as *Giardia* or *Collodictyon* are attractive candidates (Benchimol 2005, 2007; Dawson and House 2010; Zhao et al. 2012; Burki 2014). The proposed sequence of events resembles the internalization of nuclei of flagellated sperm cells by the actin-based oocytes, obvious in current eukaryotic organisms (Baluška et al. 2004a). However, the common ancestry of both histones and nucleosomes in archaea and eukaryotes strongly suggests the archaeal nature of this hypothetical guest cell that transformed into the eukaryotic nucleus-based cell body/energide assembly after entering the

host cell. This view is also supported by the fact that contemporary archaea of the TACK superphylum can have archaeal ectoparasites that develop direct contact sites with their host cells (Heimerl et al. 2017).

The host cell identity remains elusive, and because of loss of all the DNA and extensive lateral gene transfers (LGTs), it will stay so for long time. However, there are numerous strong indications for the archaeal nature of this host cell. The archaea-derived nature of host cells is reinforced by recent reports that that the TACK clade of Lokiarcheota and Asgard archaea have numerous so-called eukaryotic signature proteins that are related to both the eukaryotic cytoskeleton and endomembranes/vesicle systems (Ettema and Bernander 2009; Bernander et al. 2011; Spang et al. 2013, 2015, 2017; Nasir et al. 2015; Lindås et al. 2017; Zaremba-Niedzwiedzka et al. 2017). The TACK clade archaea contain sequences of ESCRT, TRAPP, and Sec23/24 COPII complexes; clathrin adaptors AP1–AP5, SNAREs; and small GTPases related to eukaryotic Rabs (Nasir et al. 2015; Spang et al. 2015; Klinger et al. 2016; Surkont and Pereira-Leal 2016; Rout and Field 2017; Zaremba-Niedzwiedzka et al. 2017). Importantly, Rab-like archaea proteins contain typical eukaryotic Rab motifs, structurally similar to the eukaryotic Rabs (Surkont and Pereira-Leal 2016). Moreover, the ubiquitin signaling system, crucial for eukaryotic proteome and signaling pathways, also has an archaeal origin (Grau-Bové et al. 2015). Very recently, a complex endomembrane system was reported for another member of the TACK superphylum *Ignicoccus hospitalis*, which has prompted speculation that the eukaryotic endomembrane system might also originate from archaea (Heimerl et al. 2017). Surprisingly, both the elusive host cell, named "chronocyte" by Hyman Hartman after Zeus's father Cronus (Hartman 1984; Hartman and Fedorov 2002), and the elusive guest cell, called "eocyte" by James Lake (Lake et al. 1984; Lake and Rivera 1994; Lake 2015), are also shaping up as ancient archaea. Recent advances in archaea studies suggest a new model (see table 1 in Keeling 2014), the archaean–archaean chimeric model, for the evolutionary origin of eukaryotic cells.

As evidence for ancient archaea acting as the host cell, it is also important that the TACK clade archaea not only contains the ESCRT complex but that this complex is required for the completion of cytokinesis, as is the case in eukaryotic cells (Ai and Skop 2009; Elia et al. 2011; Schiel et al. 2013; Bhutta et al. 2014; Gulluni et al. 2017; Samson et al. 2008, 2017; Liu et al. 2017). The ESCRT complex is also required for NE re-formation after mitosis (cell body/energide division) and for structural maintenance of the NE (Olmos et al. 2015, 2016; Gu et al. 2017; Denais et al. 2016; Raab et al. 2016; Vietri et al. 2016; Isermann and Lammerding 2017).

## 3.8 Nature Follows Successful Strategies: Parasitism Followed by Symbiogenesis

Nature repeats itself by duplicating successful strategies. Symbiogenesis is one of the most successful strategies of biological evolution (Kitano and Oda 2006; Douglas 2014). There are several convincing documented examples of secondary and tertiary endosymbiotic events whereby one eukaryotic cell engulfs and enslaves another eukaryotic cell (Keeling 2010; Gentil et al. 2017). Significantly, in these examples of secondary and tertiary endosymbiosis, the nuclei (known as nucleomorphs) of the enslaved cells are, as in the case of eukaryotic organelles, stripped of their DNA and genes, retaining only highly reduced genomes (Douglas et al. 2001; Moore and Archibald 2009; Keeling 2010; Gentil et al. 2017).

In several symbiotic scenarios proposed previously (Moreira and López-García 1998; Keeling 2014; López-García and Moreira 2015), the phagocytic-like origins of the eukaryotic cells prevail. But, we should also consider predatory processes not related to phagocytosis, such as the active invasion of large immobile cells by small mobile cells (Baluška et al. 2004a, b). Possibly only one invader cell takes over all of the host cell DNA and transforms itself into an ancient proto-nucleus, or perhaps several invader cells fuse together within the host cells to transform it into a proto-nucleus. Such a predatory scenario might partially solve the energetic problems associated with the phagocytototic acquisition of mitochondria. However, one cannot completely exclude the possibility that before mitochondria were acquired by the common ancestor of eukaryotic cells, these cells contained another respiratory symbiont(s) that was later fully lost, resembling the fates of mitosomes and hydrogenosomes in some eukaryotic cells. It is important to be aware that classical phagocytosis is not the only way that eukaryotic cells can internalize bacteria. For example, internalization of Rhizobia bacteria into root cells is accomplished via a process involving autophagy, although the mechanism is not well understood (Verma et al. 1991; Jones et al. 2007; Bapaume and Reinhardt 2012; Estrada-Navarrete et al. 2016). Importantly, these symbiotic Rhizobium bacteria resemble eukaryotic mitochondria in several aspects (Verma et al. 1991).

## 3.9 The Energide Strategy: Control of a Host Cell and Later Symbionts Via Stripping of Their Genomes and Coding DNA

The ultimate reason why there is no trace of genome/DNA left in the original host cell might be because the nucleus stole the genome/DNA from the endosymbionts, which were acquired by eukaryotic cells later in their evolution. For example, only about 15% of mitochondrial and plastid proteins are coded by their highly reduced genomes. The majority of their proteins are coded by the nuclear/energide genome.

Christian De Duve proposed that peroxisomes lost all of their DNA, which prevents us from conclusively proving their endosymbiotic origin (de Duve 2007). In support of this idea are mitosomes and hydrogenosomes, which are highly reduced mitochondria-like organelles of some unicellular eukaryotic organisms (Doležal et al. 2005; van der Giezen 2009; Shiflett and Johnson 2010; Zubáčová et al. 2013; Rout et al. 2016). Similarly, nucleomorphs are highly reduced relics of eukaryotic nuclei that are effectively stripped of their DNA/genomes (Moore and Archibald 2009; Keeling 2010; Archibald and Lane 2009; Grosche et al. 2014) via the master energide of the host cell. Another important aspect obscuring the evolutionary history of the eukaryotic cell is that loss of whole organelles and membranes can occur, as evidenced in examples of some tertiary and quaternary symbioses of dinoflagellates and haptophytes (Hackett et al. 2004; Archibald 2009; Qiu et al. 2013; Gould et al. 2015).

There are a variety of other kinds of evidence that all point to the neo-energide as the primary unit of life rather than the cell. The various eukaryotic organelles are remnants of once free living cells, and many bacteria and algae can exist within diverse eukaryotic cells. For example, ciliates, many invertebrates, and even vertebrate cells can host symbiotic algae (Venn et al. 2008; Kodama and Fujishima 2010; Kerney et al. 2011; Burns et al. 2017; Song et al. 2017). Moreover, in addition to mitochondria and chloroplasts, a variety of other organelles are found within cells and they all can reproduce themselves. All of them are probably the products of symbiotic cell merging that became progressively simpler over time, after they entered into a symbiotic relationship with their host cells. Cells with a single nucleus can range in size from a microscopic protist to the egg of an ostrich. Coenocytes in some species of marine algae can be several meters in length, each nucleus organizing its own set of MTs and cytoplasmic areas. A particularly dramatic example of a gigantic cell is the placenta of the developing embryo of mammals. The surface becomes highly vascularized as the villi invade the uterus to establish circulation between the embryo and the mother. It is multinucleated and the surface area can be as large as 10 $m^2$! In light of this enormous variance, might it be better to find a fundamental entity/agent that is capable of growing and dividing, and is much more uniform in its size across all the different kingdoms of life? Daniel Mazia's cell body seems to be just such a unit (Lyons 2018).

## 3.10 Final Remarks

The cell body/energide is capable of self-organization and self-reproduction and is responsive to many different external stimuli. Although the cell body/energide typically reproduces only once per cell cycle, its reproduction invariably precedes cytokinesis. Recent evidence suggests that the endosymbiotic acquisition of the eukaryotic nucleus was accomplished before that of other eukaryotic organelles and might be the first example of cooperation at a cellular level. This would explain the fact that although the timing of cell division and mitosis are tightly coordinated,

they nevertheless remain somewhat independent of each other, reflecting the symbiotic origin of the nucleus. Elucidation of the detailed molecular structures of the various membrane systems strongly supports the endosymbiotic origin of the eukaryotic nucleus. In addition, many exciting new discoveries suggest archaeal origins for both the energide and its host cell. Konstantin Mereschkovsky developed his theory of symbiogenesis as a result of his work on lichens, showing that they consisted of two organisms, a fungus and an alga, creating a symbiotic partnership. But, lichenologists disagreed. The cell theory dominated their thinking and they claimed that all living organisms were autonomous. The idea that symbiosis could be a driving force in evolution was not well received and the idea of individuality has continued to dominate biological thinking. We now know that symbiosis exists, and not only in the world of protists. Plants and animals have never been individuals; they consist not only of their own cells, but also of microorganisms whose numbers outnumber their own cell numbers. These microorganisms are crucial for normal embryonic development, for development of the immune system, and for a variety of other physical functions. The truth of the matter is that we have never been individuals: "We are all lichens" (Gilbert et al. 2012). Not only does this have profound implications for the study of development and evolution, but it also suggests that we need to rethink the idea that the eukaryotic cell is the smallest fundamental unit of eukaryotic life, and instead adopt the cell body/neo-energide concept.

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# Chapter 4
# A Brief History of Eukaryotic Cell Cycle Research


**Fatima Cvrčková**

**Abstract** The extent of literature devoted to the eukaryotic cell cycle as well as the complexity of the underlying ideas, hypotheses, and models has become rather intimidating. However, our current understanding of the processes that produce (usually) two cells out of one is rooted in a relatively limited set of underlying concepts. Some of these originated in the second half of the twentieth century, whereas others can be traced back to the early days of cell theory. Rather than striving for exhaustive coverage of all existing relevant literature, a task probably far beyond the scope of any individual, I am attempting to map the origins and historical roots of the concepts and ideas that have formed our understanding of eukaryotic cell cycle regulation. The focus is mainly on the central regulatory circuit comprising cyclin-dependent kinases and cyclins, as well as on some remarkable contributions from plant studies.

## 4.1 Introduction: The Purpose and Scope of This Chapter

As I write this essay, the term "cell cycle" is approaching half a million hits in a PubMed search (PubMed 2016). It is easy to become lost in this sea of data, terminology, interpretations, and abbreviations; in the tangled bank of concepts, models, and hypotheses that grew around the seemingly simple and intuitive notion that cells multiply by growth and division.

The cell cycle is commonly defined as the sequence of processes that produces two cells out of one by means of duplicating the mother cell's genome (DNA) and segregating it precisely between its daughter cells to produce genetically identical progeny. For now, we can leave aside special situations in which the progeny is not genetically identical because of mutations or differentiation-associated diversification as known, for example, from the mammalian immune system or from various

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cell types that lose their nuclei during differentiation (e.g., Alberts et al. 2002; Araujo et al. 2016). To make the cell lineage potentially immortal, other structural constituents should also duplicate and undergo segregation (not necessarily as precisely as the genome). In special developmental contexts, such as the early *Drosophila* embryo (Kumar et al. 2015), or in certain evolutionary lineages, such as green algae (Šetlík and Zachleder 1984; Bišová and Zachleder 2014), cells may divide their nuclei in the absence of cytokinesis and then, in a single act of division, produce more than two offspring. Even the genetic identity postulate does not hold for meiosis, which can be considered a variant of the standard mitotic cell cycle. To include these cases, we can define the cell cycle simply as the sequence of events whereby a cell gives rise to multiple, usually two, daughter cells. Here, we deal mostly with its most common version, a mitotic cell cycle producing two identical daughter cells.

This chapter attempts to guide the reader through some important landmarks of cell cycle research history—the theoretical frameworks, discoveries, and models that have shaped our current understanding of the eukaryotic cell cycle regulation. Although studies that have been awarded the Nobel Prize (Nobelprize.org 2001) can be considered as widely recognized landmarks, "landmarks" and "importance" are subjective concepts. This essay is therefore neither an exhaustive review nor a detailed science history study. If we metaphorically liken the cell cycle research field to a landscape, this is a guidebook rather than a detailed map of a territory or a geographical monograph. Other authors have produced, and will produce, diverse guidebooks (and personal travelogues) with a different focus (e.g., Hartwell 1991, 2002; Nurse 2000; Hunt et al. 2011; Duronio and Xiong 2013; Yanagida 2014; Asghar et al. 2015; Reid et al. 2015; Hunt 2015).

The structural aspects of the cell cycle, especially chromosome segregation and cell division, present a plethora of interesting problems. Ongoing research into these processes, as well as others, notably DNA replication and organelle duplication, would deserve a chapter of its own. To keep the present chapter focused, I will cover these topics only to the minimum extent necessary to consistently present the development of our understanding of the regulatory aspects.

Nineteenth century scientists had already documented mitosis and cytokinesis in astonishing detail, often based on observations in plant cells (see Sect. 4.2.1). However, the cell cycle research of the last half century has mainly been concerned with cell cycle control. Much research has been medically motivated (or at least funded by institutions concerned with biomedicine, in particular cancer studies) and therefore focused on metazoans. The introduction of non-metazoan opisthokont models such as yeast, which led to breakthrough discoveries (see Sect. 4.3.2, 4.4.1, 4.4.2 and 4.4.3), was not a trivial step because many researchers up to the 1980s doubted the existence of regulatory mechanisms shared by fungi and metazoans (Baserga 1985). Although plants were never a mainstream model for cell cycle regulation research, plant studies brought some crucial observations that shaped our view of the control of cell multiplication processes (see Sect. 4.2.2, 4.3.1, 4.4.3 and 4.5).

Because the purpose of this chapter is to illustrate rather than review, the literature coverage of the subject is, in part out of necessity, very incomplete. I sincerely apologize to all the scientists, both past and contemporary, whose

works would have deserved to be mentioned in addition to those cited, or whose results are only represented by secondary references to review articles in order to maintain a reasonable proportion between the size of this chapter and its list of references. The reader is encouraged to use the cited reviews as starting points for exploration of the primary literature.

## 4.2 Prerequisites of the Current Perspective

In the following section, I will briefly introduces three lines of inquiry underlying our current perspective. The first led to the discovery of common features of cell multiplication in various evolutionary lineages. Subsequently, the concept of the cell cycle, understood as a repetitive, precisely temporally regulated sequence of events, has been developed in parallel with (and partly inspired by) a period of intense interest in timing and periodic phenomena in other areas of the life sciences. Last, but not least, early molecular biology provided essential tools for discoveries that later resulted in the now generally accepted cell cycle models.

### *4.2.1 Cells Arise from Cells*

There could have been no notion of the cell cycle until cell theory has been established and until division was recognized as the universal mode of cell origination. It took over 170 years since R. Hooke (1665) coined the term "cell," based on observation of empty cell walls in cork, for cells to become widely acknowledged as the common constituents of living bodies in both plants and animals, mainly thanks to the works of M. Schleiden and T. Schwann in the 1830s. Further two decades elapsed before R. Virchow, building on work of his predecessors, especially Robert Remak, formulated the famous postulate that cells only come into being through division of preexisting cells (see Mazzarello 1999; Wright and Poulsom 2012).

Although M. Schleiden is usually cited for his hypothesis that cells arise de novo by "crystallization" or "precipitation" of amorphous material (e.g., Mazzarello 1999), he nevertheless clearly stated that plant cells can only originate (admittedly by a crystallization-like process) from preexisting cells. He also noticed that cells are born small and subsequently enlarge (Schleiden 1838). Plant cell boundaries are easier to observe than those in metazoan tissues; therefore, it is not surprising that the first observations of cytokinesis were made in representatives of the plant kingdom, algae and mosses (Mohl 1835 and references therein; see also Paweletz 2001). Soon thereafter, cell plate formation was described in monocot root tips (Nägeli 1842). By the mid-nineteenth century, the possibility that cells are generated de novo remained a heavily disputed minority hypothesis (reviewed in Remak 1852), although it lingered in the literature until the 1870s (Paweletz 2001).

Convincing observations of metazoan cells dividing by constriction were reported by Remak (1852).

Although "dissolving" and reconstitution of plant cell nuclei prior to cell division was already recognized by K. Nägeli (1842), description of mitosis, made possible by progress in microscopy methods and instrumentation, is attributed mainly to the works of A. Schneider and W. Flemming in the 1870s (Paweletz 2001). In the following decade, E. Strasburger, better known as the founder of an influential series of plant biology textbooks, morphologically characterized the process of mitosis in detail and introduced the terminology for mitotic stages that is still in use (Baluška et al. 2012).

Following the 1900 rediscovery of Mendel's laws (see Šimůnek et al. 2011), the biological significance of mitosis became obvious after the influential geneticist T.H. Morgan embraced the disputed theory of chromosomes as the physical residence of genes (Benson 2001). Leaving aside the sad chapter of "Soviet creative Darwinism" (Rapoport 1991), mitosis was generally acknowledged by the mid-twentieth century as a common, if not universal, mode of eukaryotic nuclear division, although observations of "direct nuclear division" or "amitosis" are still being sporadically reported. Some of these cases are either genuine division of amplified macronuclear chromatin in ciliates whose germline micronucleus divides mitotically (Ruehle et al. 2016) or processes unrelated to cell division, such as nuclear fragmentation in terminally differentiated or dying cells, designated amitosis out of terminological inertia (e.g., Wang et al. 2010). Relevant for the history of cell cycle research, yeasts were suspected to divide by amitosis well into the mid-twentieth century, because their mitotic chromosomes do not become condensed and chromosome segregation is not accompanied by disintegration of the nuclear envelope. Geneticists strove in vain to detect mitotic chromosomes in the model budding yeast, which was already known to exhibit Mendelian inheritance, up to the point of occasionally reporting experimental artefacts or vacuolar inclusions as "chromosomes" (see Hall et al. 1993), until closed mitosis of yeast cells was recognized as a variant of standard mitosis (reviewed in Boettcher and Barral 2013). By the 1960s, the sequence of events taking place during standard mitotic division in all three lineages whose members commonly served as model organisms (metazoans, plants, and yeasts) had been, in principle, well established (Yanagida 2014).

### *4.2.2 Rhythm of the Mitotic Dance*

Leaving differentiation and cell death aside, cells can be either quiescent (nondividing) or alternate between two morphologically distinct states: the interphase, where they are seemingly "doing nothing", and mitosis, usually followed by cytokinesis. In the early 1950s, several groups simultaneously reported that the amount of a cell's DNA, now known to be the genetic material (Avery et al. 1944), doubles during a distinct temporal window within interphase (see Pedersen 2003).

Quantitative microscopy in metazoan cells (Swift 1950; Walker and Yates 1953) and experiments with radioactive DNA labeling in plants (Howard and Pelc 1951) led to the recognition of the "standard" eukaryotic cell cycle (or, more precisely, nuclear cycle) consisting of four phases: G1, S, G2, and M, with a fifth phase (G0) usually added to describe a quiescent or out-of-cycle state (Fig. 4.1). Methods utilizing labeled precursors were employed to study the timing of individual cell cycle phases (e.g., Thrasher 1966). These works produced the general notion that the duration of phases is relatively constant in cell populations undergoing steady-state renewal, defined by population properties (except its size) being constant in time, a situation analogous to what is denoted as "balanced growth" in microbiology (Schaechter 2015). Because cells usually take longer to duplicate their mass than to divide their essential components, the nuclear cycle events (genome replication and segregation) must be controlled to keep the cell population properties stable (Mitchison 1971, 2003).

Bacteriology has been a constant source of observations, methodologies, and theoretical approaches that have inspired research into eukaryotes. Remarkably, the bacterial cell cycle was long considered fundamentally different from that of eukaryotes, since rapidly growing bacteria, such as the common models *Escherichia coli* and *Bacillus subtilis*, seemingly replicated DNA continuously rather than exhibiting a distinct S phase due to their ability to initiate a new round of chromosome replication prior to finishing the previous one (reviewed in Wang and Levin 2009). This feature, known as multifork replication, is possible because bacteria only have a single replication origin within their circular chromosome, and because prokaryotic cell organization allows for gene expression throughout the cell cycle, whereas expression of many (though far from all) eukaryotic genes ceases during mitosis when chromatin is condensed (Chen et al. 2005). Nevertheless, by the 1970s, bacteriological studies had produced essential

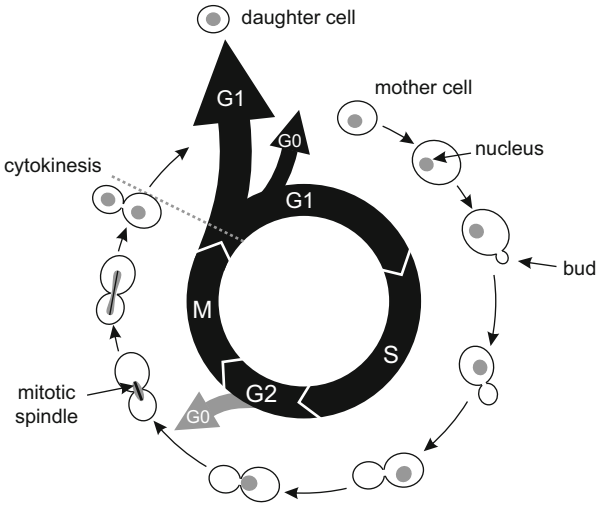


**Fig. 4.1** Inner circle: Phases of generic eukaryotic cell cycle. Outer circle: characteristic phenotype of budding yeast cells in distinct cell cycle phases. Note that budding yeast, as a rule, exits the cycle into G0 only from the G1 phase and the exit route from G2 is therefore shown in gray

techniques that were also applicable to eukaryotic microbes (such as yeasts) and to cell cultures derived from multicellular organisms.

Methods for synchronizing cell populations (see Helmstetter 2015) and analyzing their age composition based on incorporation of tagged compounds allowed temporal mapping of cell cycle events. In addition, the larger eukaryotic cells were also amenable to direct microscopic observation of cell cycle progress, a feature utilized by researchers ever since, nowadays with the aid of sophisticated techniques for in vivo tagging of intracellular structures (Henderson et al. 2013). An influential summary of the classical studies using these methods, in conjunction with biochemical and pharmacological techniques, was published by J. Murdoch Mitchison (1971, 1974), the founder of a major research school in Edinburgh that established the fission yeast *Schizosaccharomyces pombe* as a mainstream model organism for cell cycle studies.

Alan Turing's description of a simple hypothetical mechanism capable of generating repetitive spatial structures (Turing 1952) and the discovery of the Belousov–Zhabotinsky chemical oscillator (see Winfree 1984) initiated a period of increased interest in periodic processes and repetitive structures in many areas of the natural sciences during the 1950s to 1970s. Sensitive methods of biochemical analysis allowed observation of metabolic oscillations, which became the subject of intensive study (summarized in Goldbeter 1997). Research into diurnal periodicity in the behavior and physiological functions of various organisms culminated in 1971 in the isolation of the first *Drosophila* mutants with a defective circadian clock (see Loudon et al. 2000). The perspective of the cell cycle as a temporally regulated sequence of events, also largely established during the 1950s and 1960s, fitted well into the general scientific context of the time, and naturally raised the question of how the timing of these events is controlled. In other words, what determines the rhythm of the mitotic "dance of the chromosomes" (Walczak et al. 2010) and other essential cell cycle processes such as genome replication and cytokinesis?

### *4.2.3 Self-Assembling Machines*

The 1950s and 1960 were also the era that established molecular biology as a methodological approach, if not yet a scientific field studying the forms, evolution, and function of biological molecules, including their contribution to higher levels of organization within living cells (Astbury 1961). One of the earliest major achievements of this approach was the reconstitution of infectious particles of the rod-like tobacco mosaic virus from purified protein and RNA (Fraenkel-Conrat and Williams 1955; further work summarized by Fraenkel-Conrat 1970). Reconstitution of other viruses, including structurally complex tailed bacteriophages such as R17 (Roberts and Steitz 1967) followed.

Macromolecular assemblies of cellular origin, including ribosomes, have also been successfully reconstituted in vitro (Traub and Nomura 1968, 1969; Kushner

1969). These early studies resulted in a wave of somewhat unrealistic optimism that the properties of supramolecular assemblies can be, at least as a rule, fully derived from those of their parts. Nevertheless, it should be stressed that contemporaries did admit the possibility of mechanisms other than physicochemically determined self-assembly contributing to cell organization (e.g., Kushner 1969). This, in turn, boosted interest in studying the assembly of subcellular structures in vivo.

In vitro reconstitution experiments were soon complemented by utilization of conditional, for instance temperature-sensitive, mutants unable to complete specific steps in generating the macromolecular assembly of interest (e.g., a phage particle) under certain conditions (Groman 1962; Edgar and Lielausis 1964). Directly relevant to our topic, an analogous approach, based on isolation and characterization of temperature-sensitive mutants with defects in ribosome assembly or function (manifested as an abrupt inhibition of protein synthesis at the restrictive temperature), was applied by Leland H. Hartwell and coworkers to dissect the molecular mechanism of protein synthesis in a model unicellular eukaryote, the budding yeast *Saccharomyces cerevisiae* (Hartwell and McLaughlin 1968, 1969; Hartwell et al. 1970b). Some of the mutants obtained within this project were later central for a breakthrough genetic study of the yeast cell cycle (see Sect. 4.3.2).

## 4.3 Dominoes and Clocks: Two Views of Cell Cycle Control

Much of the cell cycle research of the 1970s and 1980s attempted to answer a crucial question posed by J.M. Mitchison (1971): Is the temporal organization of cell cycle events determined by a central regulatory system (a "timer" or "clock") or can the (usually) fixed order of cell cycle steps be explained by causal dependence of certain events of the cell cycle on the completion of previous events? The latter "falling domino" model implies that cell cycle regulation can be mapped similarly to, for example, the succession of intermediates and enzyme-catalyzed steps in the classical pathways of intermediary metabolism (Fig. 4.2; see, e.g., Hartwell et al. 1974). Research aiming to identify the components of the central clocks, or to provide evidence supporting the domino model, progressed along mutually independent lines for most of two decades.

### *4.3.1 Evidence for Central Control of Cell Cycle Timing*

The mammalian cell fusion experiments of B.P. Rao and R.T. Johnson (1970), documenting that exposure to S phase cytoplasm can induce DNA synthesis in G1 but not in G2 nuclei, are often cited as the first demonstration that nuclear events of the cell cycle are controlled by the cytoplasm (e.g., Yanagida 2014). However, their authors were well aware of earlier observations in slime mold plasmodia (see Johnson and Rao 1971), where cytoplasmic factors were shown to regulate the

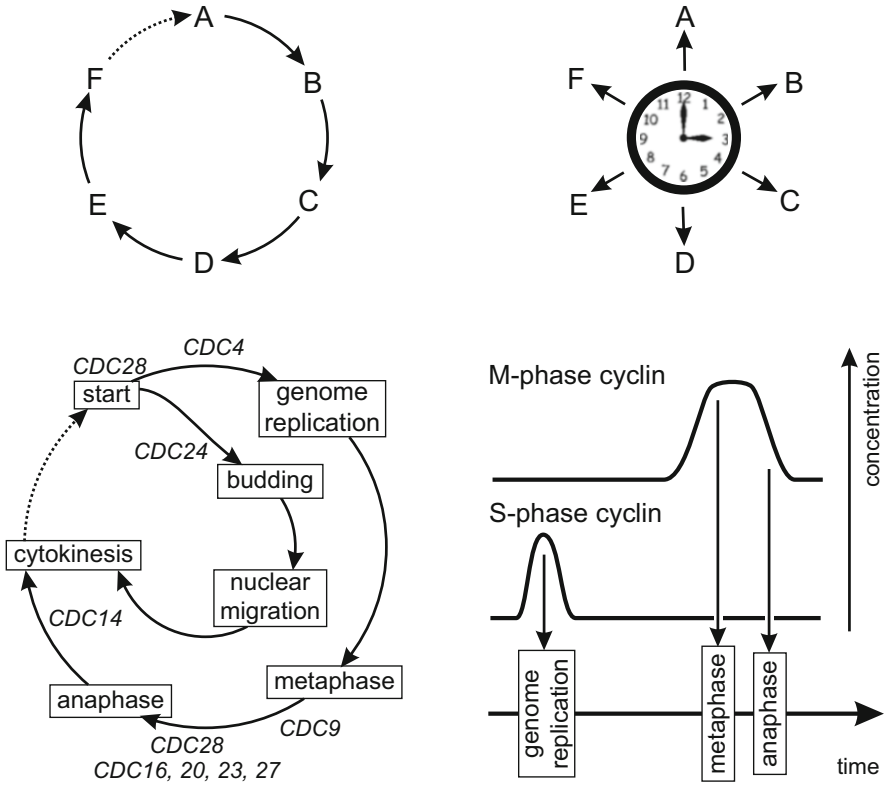


**Fig. 4.2** Top: Scheme of generic "domino" (left) and "clock" (right) models of controlling the order of cell cycle events A through F. Bottom: Example of a domino-type sequence of steps in the budding yeast cell cycle controlled by distinct *CDC* gene products (left; modified from Hartwell et al. 1974; see Table 4.1 for the genes shown), and a simple clock-type control mediated by CDK–cyclin complexes whose activity and specificity depends on the type and concentration of cyclin(-s) present (right; note that anaphase is triggered by mitotic cyclin degradation)

onset of mitosis (Rusch et al. 1966). Experiments with grafting ciliate cytoplasm suggested cytoplasmic control of nuclear division a decade earlier (see Duesbery and Vande Woude 1988). Synchronous nuclear division in syncytial tissues such as the endosperm of some angiosperm plants has been documented at least since the beginning of the twentieth century (Wilson 1902). Although maize endosperm was routinely used as a source of synchronous mitotic spindles for morphological studies as early as the 1950s (Duncan and Persidsky 1958), concerted nuclear division in plant syncytia was not exploited to study cell cycle regulation until after the first metazoan cell fusion studies. Also later observations of nuclear cycle synchrony in multinucleated plant protoplasts were reported in a descriptive manner (Fowke et al. 1975). A decade after the hallmark mammalian cell study of Rao and Johnson (1970), analogous results were published for plant cells (Szabados and Dudits 1980), indicating that the cytoplasmic factors

determining cell cycle phase, even if not necessarily homologous, work in a similar manner in metazoans and plants.

The path toward biochemical and molecular characterization of cytoplasmic regulators of cell cycle events began with the discovery of a "maturation promoting factor" (MPF) by Yoshio Masui and Clement Markert (1971). MPF was originally detected as a complex cytosolic fraction from frog oocytes induced to mature (i.e., finalize the second meiotic division) by progesterone treatment. An analogous activity was also present in cleaving embryonic cells and could trigger oocyte maturation when certain cytosolic fractions were injected into noninduced oocytes. Analogous activity peaking during each cell cycle was soon discovered in other dividing cell populations, including invertebrate embryos, mammalian cell cultures, and even yeast (reviewed by Duesbery and Vande Woude 1988; Masui 2001). It took nearly a decade before active MPF was partially purified and hypothesized to possess protein kinase activity (Wu and Gerhart 1980). Its biological activity was later found to alternate with that of an "MPF inactivating agent" of unknown nature (Gerhart et al. 1984). Further biochemical purification of MPF confirmed its protein kinase activity and demonstrated that this activity requires two polypeptides of 32 kDa and 45 kDa (Lohka et al. 1988).

In the meantime, a crucial clue for understanding the cause of the periodic behavior of MPF came with the discovery of cyclins by the research team led by Tim Hunt (Evans et al. 1983; Pines and Hunt 1987). Cyclins were originally defined as a family of mutually related proteins whose intracellular concentrations during each cell cycle gradually increase and then abruptly decrease. For some of them, the concentration maximum coincided with the peak of MPF activity. We now know that certain cyclin subfamilies peak at different cell cycle stages (e.g., at the onset of S phase) and that some cyclins do not exhibit periodic concentration changes at all despite a clear sequence homology to their periodically behaving relatives (Minshull et al. 1989; for a recent view of cyclin diversity see Ma et al. 2013). Unfortunately, the term "cyclin" has been previously used for what is now known as the proliferating cell nuclear antigen (PCNA), a DNA binding protein whose abundance in a cell population reflects its ability to proliferate (Bravo et al. 1982; Matsumoto et al. 1987) and which is not related to the above-mentioned protein family. This resulted in long-lasting terminological confusion. In violation of the generally accepted priority principle of biological terminology, but in agreement with common current usage, here I use the term "cyclin" solely to describe members of the protein family first identified by Evans et al. (1983) based on periodic cell cycle phase-dependent changes in the abundance of some of its members.

Injection of heterologous (mollusc) cyclin-encoding mRNA into frog oocytes mimicked the effect of MPF (Swenson et al. 1986). Using an ingenious cell-free frog oocyte extract system, de novo cyclin translation was later found to be both necessary and sufficient for acquisition of the ability to trigger chromosome condensation in added sperm nuclei (Murray and Kirschner 1989a), consistent with a cyclin being the activity-limiting component of the MPF. Although attempts to link cyclins directly to the MPF complex were carried out during most of the late

1980s (see Hunt 2002, 2015), final connecting evidence came from an independent line of research in yeast genetics discussed in the next section.

### 4.3.2 The Domino Model: Cell Cycle as a Sequence of Interdependent Events

The identification and characterization of protein synthesis-defective temperature-sensitive mutants of the budding yeast *S. cerevisiae* (see Sect. 4.2.3) was the first tangible result of a large screen designed in the late 1960s by L. Hartwell, originally a phage geneticist, with the aim to identify genes required for structural cell cycle events such as DNA replication or mitosis. His experimental strategy, inspired by the approach previously used to dissect the life cycle of several bacteriophages (Reid et al. 2015), was based on isolation of so-called cell division cycle (*cdc*) mutants (i.e., temperature-sensitive mutants defective in genes required for distinct cell cycle events). In agreement with the standard terminology of budding yeast genetics, I refer to the (usually dominant) wild-type alleles of these genes as *CDC* genes, and use italics to describe genes and alleles, while plain-text abbreviations with first capital letter refer to proteins.

The stage of the budding yeast cell cycle can be easily inferred by simple microscopic observation, because (under normal conditions) bud emergence coincides with the onset of genomic DNA replication, and migration of the nucleus to the bud neck takes place at the beginning of mitosis (Fig. 4.1). Thus, *cdc* mutants at a nonpermissive temperature become arrested with a characteristic "terminal phenotype" that may correspond to a normal cell cycle stage (e.g., cells arrested at G1 prior to the onset of budding and DNA synthesis, such as most *cdc28* mutant alleles), but in other cases deviates from normal cell organization. For example, *cdc24* cells cannot bud at the restrictive temperature but become multinucleated, indicating that nuclear events can proceed even in the absence of cell division. On the other hand, *cdc4* mutants produce multibudded cells with a single nucleus arrested in G1, an observation difficult to reconcile with a strict domino model but compatible with the presence of a central "clock" (Hartwell et al. 1974).

Over 30 *CDC* genes whose mutation resulted in cell cycle arrest at 37 °C (a temperature at which wild-type *S. cerevisiae* can still grow) were identified in the original screen. Apart from the rare exception of an anomalous *cdc28* allele (see below), different mutant alleles of any given *CDC* gene exhibited a consistent, gene-specific (i.e., not allele-specific) terminal phenotype, and mutant cells continued to grow in size at the nonpermissive temperature, resulting in abnormally large cells (Hartwell et al. 1970a, 1973; further work summarized by Hartwell 1991, 2002; Reid et al. 2015). A combination of time-lapse microscopy, inhibitor studies, and genetic techniques was used to map the network of dependencies between cell cycle steps requiring individual *CDC* genes (i.e., the order of the metaphorical falling domino blocks). This sequence of *CDC* gene functions turned out to

bifurcate into two distinct pathways at or after the stage controlled by *CDC28*, with one branch encompassing nuclear events (DNA replication and mitosis) and the other cytoplasmic ones, i.e. budding, nuclear migration, and cytokinesis (Hartwell et al. 1973, 1974; Fig. 4.2).

*CDC28* appeared to be exceptional for additional reasons. First, *cdc28* mutants were the only *cdc* mutants that could enter the sexual process (a developmental trajectory alternative to the standard mitotic cell cycle) while arrested at the restrictive temperature. This suggested that the step controlled by *CDC28*, which has been termed the "Start" of the cell cycle, might correspond to a point of commitment to enter the cycle (Hartwell et al. 1974). Second, an unusual temperature-sensitive mutant allele of *CDC28*, which arrested at mitosis rather than at Start, was isolated later, indicating that, unlike other *CDC* genes, *CDC28* is involved in multiple stages of the cell cycle (Piggott et al. 1982).

Inspired by L. Hartwell's experiment, Paul Nurse, who started working with the fission yeast *S. pombe* during a postdoctoral stay in the laboratory of J.M. Mitchison, performed a screen for *cdc* mutants in *S. pombe*, resulting in the identification of 14 fission yeast genes whose mutations resulted in a *cdc* phenotype (Nurse et al. 1976). At the time, detection of possible homology between known budding yeast *CDC* genes and the new cell cycle genes from fission yeast was far from straightforward. Cloning by complementation was only introduced several years later; in a remarkable coincidence, the first yeast gene successfully isolated by this method was budding yeast *CDC28* (Nasmyth and Reed 1980).

As an unfortunate consequence, the terminology of *cdc* mutants developed independently in budding and fission yeast, and there is thus no consistent relationship between *cdc* gene numbering in these two organisms (see Table 4.1). However, the possible confusion is at least in part mitigated by species-specific terminological conventions: *S. pombe* genes are usually labeled by lowercase letters with additional allele indication in superscript (e.g., $cdc2^{+}$ is a wild-type allele, whereas $cdc2^{ts1}$ is a mutant one; as in the case of budding yeast, proteins are denoted in plain text with the first letter in capitals).

Apart from mutants exhibiting characteristic *cdc* features (conditional cell cycle arrest and increased cell size at restrictive temperature), the fission yeast screen yielded some mutants with conspicuously small cells, indicative of an alteration in the control of cell division (Nurse 1975; see also Nurse 2002, 2016). Some of these small cell mutants defined a dominant allele of $cdc2^{+}$, whose recessive alleles caused cell cycle arrest in either G2 or G1, with the G1-arrested cells able to conjugate (Nurse and Bissett 1981). Fission yeast $cdc2^{+}$ thus exhibited several features remarkably similar to budding yeast *CDC28*. Indeed, a fission yeast temperature-sensitive mutant ($cdc2^{ts}$) was complemented by expression of the budding yeast *CDC28* gene (Beach et al. 1982). Subsequent sequence analysis showed that *S. cerevisiae CDC28* and *S. pombe* $cdc2^{+}$ encode closely related, homologous protein kinases (Lörincz and Reed 1984; Hindley and Phear 1984). Thus emerged an overall picture of the yeast cell cycle regulated by a group of

**Table 4.1** Overview of yeast *CDC* genes mentioned in this chapter

| *S. cerevisiae*[a] | *S. pombe* | Product, function, or phenotype |
|---|---|---|
| *CDC4* | *pop2*$^+$ | Subunit of a specific E3 ubiquitin ligase complex involved in G1/S cyclin degradation; mutants fail to enter S phase while budding continues; see Sect. 4.4.2 |
| *CDC9* | *cdc17*$^+$ | DNA ligase; mutant cell cycle is arrested as a result of triggering a checkpoint pathway by DNA breaks; see Sect. 4.4.3 |
| *CDC14* | *clp1*$^+$ | Protein phosphatase implicated in the control of cytokinesis; see Sect. 4.4.3[b] |
| *CDC16* | *cut9*$^+$ | Subunit of a specific E3 ubiquitin ligase complex, the anaphase promoting complex (APC); mutants arrest at G2/M; see Sect. 4.4.2[c] |
| *CDC20* | *slp1*$^+$ | One of two alternative regulatory subunits of the APC; mutants arrest at G2/M; see Sect. 4.4.2[c] |
| *CDC23* | *cut23*$^+$ | Subunit of the APC; mutants arrest at G2/M; see Sect. 4.4.2[b] |
| *CDC24* | *scd1*$^+$ | Cofactor of a RHO-clade small GTPase required for bud formation; mutants fail to bud while the nuclear cycle continues; *S. pombe* homolog is involved in cytokinesis[d] |
| *CDC27* | *nuc2*$^+$ | Subunit of the APC; mutants arrest at G2/M; see Sect. 4.4.2[c] |
| *CDC28* | *cdc2*$^+$ | Cyclin-dependent kinase (CDK); see Sect. 4.3.2 |
| Multiple | *cdc13*$^+$ | Cyclin; in budding yeast cyclin mutations do not result in a *cdc* phenotype because of functional overlap between related proteins |
| *MIH1* | *cdc25*$^+$ | Protein phosphatase responsible for removing inhibitory phosphorylation of the Cdc2 kinase; two functionally overlapping homologs in budding yeast; mutation in fission yeast results in G2 arrest; see Sect. 4.4.3[e] |

[a]A complete summary of classical budding yeast *CDC* genes can be found in Reid et al. (2015)
[b]For fission yeast homologs, see Trautmann et al. (2004)
[c]For summary of fission yeast APC subunits, see Pines (2011)
[d]Li and Chang (2003)
[e]For budding yeast homologs, see Sia et al. (1996)

genes whose products act in sequence, with the conserved Cdc2/Cdc28 protein kinase participating in the regulation of several cell cycle steps.

## 4.4 The Universal Model of Cell Cycle Control

The reconciliation (and ultimate merging) of the seemingly incompatible clock and domino models took nearly two decades (Murray and Kirschner 1989b). The resulting unified model of the cell cycle has since become not only a well-established part of the standard molecular biology paradigm, firmly anchored in textbooks (e.g., Alberts et al. 2002), but also the starting point for much of the ongoing cell cycle research.

### 4.4.1 Reconciling the Domino and Clock Models

Several breakthrough discoveries at the end of 1980s led to molecular identification of the key components of the MPF as homologs of products of yeast *CDC* genes. A human cDNA encoding a protein kinase homologous to the product of *CDC28*/*cdc2*$^{+}$ was cloned by complementation of a fission yeast *cdc2*$^{ts}$ mutation (Lee and Nurse 1987). Antibodies raised against this kinase cross-reacted with the 32 kDa protein, a key component of the MPF (see Sect. 4.3.1; Gautier et al. 1988). Around the same time, the sequence of *cdc13*$^{+}$, a fission yeast gene shown to interact genetically with *cdc2*$^{+}$, was found to be related to metazoan cyclins (Booher and Beach 1988; Hagan et al. 1988; see also Hunt 2015 for an interesting personal reminiscence related to these discoveries).

By the end of the 1980s, it became clear that organisms as diverse as yeasts, sea urchins, frogs, and mammals all possess at least one Cdc28-related protein kinase (a cyclin-dependent kinase; CDK) and, as a rule, multiple cyclins. However, the inventory remained far from complete for more than a decade, even in well-established models such as the budding yeasts, whose full genomic sequence was reported only in 1996 (Goffeau et al. 1996). Curiously, no cyclin was found in L. Hartwell's classical screen for *cdc* mutants in *S. cerevisiae*. We now know that this was due to an extensive functional overlap or "redundancy" between the nine budding yeast cyclins (Nasmyth et al. 1991; Reid et al. 2015). Nevertheless, even an incomplete inventory was sufficient to justify a general model in which the cell cycle phase is determined by the repertoire of active CDKs present at any given moment, and in which CDK activity is controlled by cyclins whose levels periodically fluctuate as a result of regulated protein synthesis and degradation (Minshull et al. 1989; Fig. 4.2).

Although plant cell cycle research of the 1980s and 1990s lagged considerably behind that in metazoans and yeasts, by the mid-1990s it became clear that the general principles of cell cycle control by CDKs and cyclins also hold for plants (see Day and Reddy 1994; Segers et al. 1996; Renaudin et al. 1996 and references therein). Further research supported the validity of the model of cell cycle control by CDKs and cyclins for all eukaryotes. The model developed into one of the major paradigms of current cell biology and resulted in a well-deserved Nobel Prize, awarded in 2001 to L. Hartwell, T. Hunt, and P. Nurse (Nobelprize.org 2001). The notion of cell cycle control by CDKs and cyclins paved the way for research into molecular mechanisms controlling cyclin abundance and modulating CDK activity. A substantial part of cyclin abundance regulation takes place at the transcriptional level. Although molecular details may differ in yeast, metazoans, and plants, positive and negative feedback loops are a common feature of transcriptional control of cell cycle-regulated genes, including those encoding cyclins themselves (e.g., Koch and Nasmyth 1994; Bertoli et al. 2013). Interestingly, both CDKs and cyclins exhibit significant protein sequence similarity with several proteins of the eukaryotic transcription apparatus, and some CDK isoforms (e.g., mammalian Cdk7) directly participate in transcription control (see Sansó and Fisher 2013; Malumbres 2014). The cell cycle regulators may thus have evolved through specialization of a preexisting family of transcriptional regulators.

Equally important as tightly controlled cyclin production is the timely removal of these regulatory proteins by specific proteolysis, which will be discussed in Sect. 4.4.2.

Remarkably, selective dephosphorylation of CDK substrates does not seem to be a decisive factor in cell cycle control apart from some special cases, which, however, may be of extreme biological importance. For example, the evolutionarily conserved retinoblastoma protein pRB is a master regulator of cell cycle progression-related transcription. Its activity is controlled by a complex "phosphorylation code" resulting both from CDK-dependent phosphorylation and specific dephosphorylation (Rubin 2013). Other examples of selective dephosphorylation implicated in cell cycle control include the protein phosphatases encoded by *cdc25*$^{+}$ (see below) and *CDC14* (see Sect. 4.4.3). However, specific proteolysis appears to be the main mechanism ensuring removal of phosphorylated CDK substrates that have fulfilled their function.

CDKs are also regulated, both positively and negatively, by phosphorylation at distinct tyrosine and threonine residues (Lorca et al. 1992; for further work see Nurse 2002). In the fission yeasts, the balance of inhibitory tyrosine phosphorylation (mediated by the product of the *wee1*$^{+}$ gene) and dephosphorylation by the *cdc25*$^{+}$-encoded phosphatase regulates cell size by delaying entry to mitosis until the critical cell size is reached (see Nurse 2002). An analogous regulatory circuit also operates in budding yeast and in metazoans, although it is used to control entry into mitosis in different physiological contexts (Bouldin and Kimelman 2014; see also Sect. 4.4.3). Higher plant *cdc25*$^{+}$ homologs diverged substantially from the yeast and metazoan ones and lack the ability to complement the fission yeast *cdc2*$^{ts}$ mutation. Nevertheless, plants have retained regulatory phosphorylation at the conserved substrate site, which may contribute to cell cycle control under specific developmental or physiological circumstances (Francis 2011).

In addition to activation by cyclins, CDK activity is also negatively regulated by a heterogeneous collection of inhibitory subunits, collectively termed CDK inhibitors (CKIs). Many of these were originally discovered as tumor suppressor genes (e.g., Koff and Polyak 1995; Woollard et al. 1996; Vidal and Koff 2000; Cánepa et al. 2007). CKIs are often involved in modulating cell cycle control in response to extracellular (e.g., hormonal or growth factor) signals, but they can also participate in developmental decisions, including those resulting in modification of the cell cycle itself such as, e.g., genome endoreduplication (see Sect. 4.5).

### 4.4.2 *Making the Clock Tick: Mechanisms Ensuring Periodic Behavior*

The above-outlined model of cell cycle control raises an important question: What ensures that waves of individual CDK/cyclin complex activities follow each other in an orderly and periodic fashion? This could, in principle, be achieved solely by transcriptional regulation of cyclin-encoding genes, assuming that cyclin proteins

are inherently unstable and that cyclins active in a particular phase auto-activate their own transcription and repress that of other cyclin-encoding genes whose activity is undesirable at a given cell cycle stage (Amon et al. 1993). However, experiments in budding yeast showed that regulated cyclin proteolysis plays an important role in preventing accumulation of cyclins at an improper time (Amon et al. 1994). Cyclin degradation at the G2/M and metaphase/anaphase transitions is controlled by a specific E3 ubiquitin ligase complex, the anaphase promoting complex (APC) or cyclosome, which contains products of several genes identified in L. Hartwell's original *cdc* screen, namely *CDC16*, *CDC23*, *CDC26*, and *CDC27*. APC uses distinct regulatory subunits for its two phases of activity; one of them is encoded by *CDC20* (see Peters 1999; Pines 2011; Reid et al. 2015). Another specific E3 ubiquitin ligase complex, containing (among others) the product of the *CDC4* gene, drives degradation of G1/S-specific cyclins and several other proteins that undergo periodic proteolysis depending on their CDK-mediated phosphorylation after S phase entry (Toda et al. 1999; Willems et al. 1999).

Identification of the regulatory circuits responsible for periodic oscillations of the CDK/cyclin complex activities has enabled the use of theoretical and mathematical models for testing hypotheses, as well as for generating evolutionary scenarios that might have given rise to a minimum set of cell cycle regulators capable of sustained and robust periodic oscillations (e.g., Novák et al. 1998; subsequent work reviewed in Tyson and Novák 2008, 2015; Uhlmann et al. 2011). Some of these models predicted the existence of regulatory components prior to their experimental discovery (see Csikász-Nagy 2009), or provided an explanation for otherwise puzzling observations such as the relatively constant duration of mitosis compared to other cell cycle phases (Araujo et al. 2016).

Early modeling efforts highlighted an underlying similarity between the minimal cell cycle oscillator (consisting of a CDK/cyclin complex inducing its own amplification and an APC activated in response to CDK and bringing about its inactivation; see Fig. 4.3) and other previously characterized biological oscillators, in

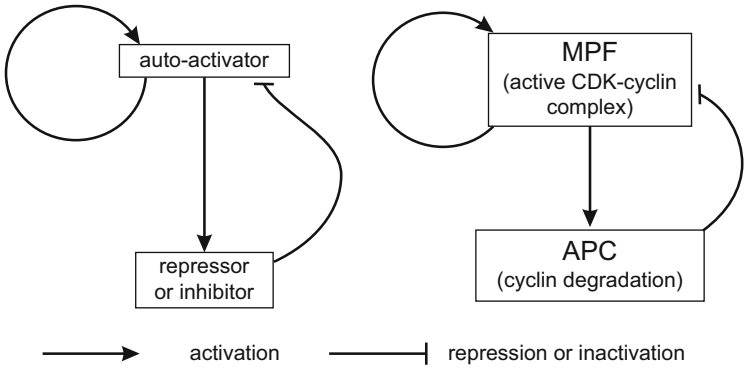


**Fig. 4.3** Left: Scheme of a generic regulatory circuit capable of sustained robust oscillations. Right: The simplest implementation of this generic oscillator in cell cycle control (modified from Ingolia and Murray 2004)

particular the circadian clock (Ingolia and Murray 2004; see Sect. 4.2.2). Both the cell cycle oscillator and the circadian clock are representatives of a negative feedback loop with amplification, one of several simple regulatory architectures that generate robust, sustained oscillations over a wide range of parameters (Novák and Tyson 2008).

The current image of the eukaryotic cell cycle as a series of events controlled by a central oscillator or clock, in the sense first proposed by J.M. Mitchison (1971), thus emerged. The inner workings of this clock, nowadays understood in much molecular detail and successfully emulated by mathematical models, can be described as a network of regulatory interactions based on transcription, posttranslational protein modification, protein complex formation, and targeted degradation. These processes, in turn, can be described in terms of domino-type models.

### *4.4.3 Inputs and Outputs of the Central Oscillator*

The above-outlined view of cell cycle regulation raises the following questions: How is the central cell cycle clock connected to inputs, both extracellular (e.g., hormone signals or nutrient status) and intracellular (e.g., cell size, perception of genomic damage)? How does this clock control the structural events of the cell cycle such as DNA replication or genome segregation (Fig. 4.4)?

The first answers to these questions came with the characterization of *cdc9*, a classical budding yeast *cdc* mutant arresting at the restrictive temperature with a post-replication (G2) nucleus located at the mitotic position at the bud neck. Surprisingly, *CDC9* turned out to encode DNA ligase, which is necessary for repairing DNA breaks that arise naturally in the process of lagging DNA strand replication (Johnston and Nasmyth 1978). Mutations of the homologous fission yeast gene *cdc17*$^{+}$ also lead to conditional cell cycle arrest (Barker et al. 1987). The

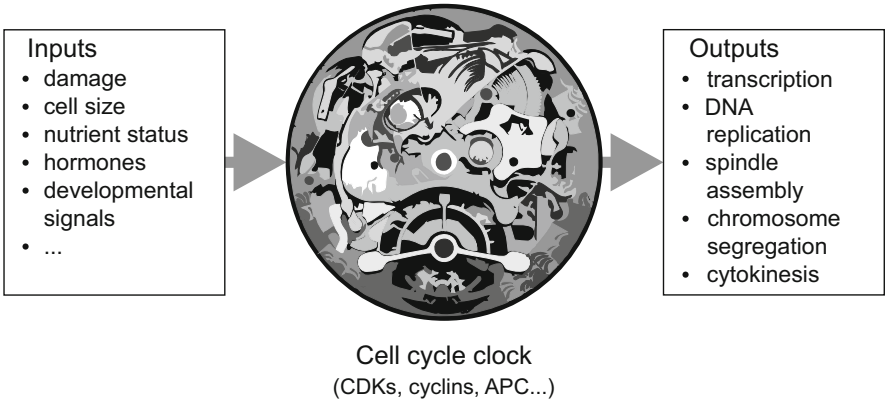


**Fig. 4.4** The central "cell cycle clock" with its inputs and outputs

cell cycle arrest in *cdc9* mutants at the restrictive temperature is caused by a DNA damage-triggered signaling pathway involving the *RAD9* gene product (Schiestl et al. 1989; Weinert and Hartwell 1990). Thus was discovered the first "checkpoint control" mechanism that can block cell cycle progression in cases of damage to the genetic material or failure of structural cell cycle events. *RAD9* is evolutionarily conserved and acts at least in part by activating transcription of CDK inhibitors (Lieberman and Yin 2004).

In addition to mediating cell cycle arrest in response to DNA damage, the *RAD9*-dependent pathway also prevents cell cycle progression beyond G2 until genome replication is completed (Weinert 1992). An additional partly conserved checkpoint pathway, which was discovered later in yeasts, metazoans, and plants, can block the cell cycle if a chromosome fails to attach to the mitotic spindle (see Musacchio 2015; Komaki and Schnittger 2016). Failure of cytoplasmic events of the cell cycle may also trigger a checkpoint pathway, because *S. cerevisiae* cells unable to form a bud temporarily arrest the cell cycle by a mechanism involving inhibitory CDK phosphorylation (Lew and Reed 1995; Sia et al. 1996). The phosphorylation takes place at a site known to mediate the coordination between growth and division in fission yeast (compare Sect. 4.4.1), hinting at lineage-specific diversity of inputs controlling the central oscillator.

Besides damage, developmental signals mediated by cell-to-cell contacts, diffusible substances, or nutrient status can also modulate cell cycle progression. The signaling pathways regulating cell cycle entry are diverse and generally lineage-specific, as illustrated, e.g., by a recent systematic comparison of cell cycle regulators in opisthokonts and the green alga *Chlamydomonas reinhardtii* (Tulin and Cross 2014). The input pathways often converge on transcription of G1-specific cyclins (for a review of the situation in mammals and budding yeast, see Duronio and Xiong 2013; Fisher 2016). However, cells of many organisms can also (or even predominantly) exit the cell cycle in G2, and external signals or cell size can thus regulate entry into mitosis. This is the case in the fission yeast (Sveiczer and Horváth 2016), as well as in plants (Gutierrez 2016; Magyar et al. 2016).

Less diverse than the inputs regulating the cell cycle oscillator are its outputs. Barring unusual cases, such as cell cycles with postponed cytokinesis producing multiple progeny (see Sect. 4.1.) or genome endoreduplication (see Sect. 4.5.), every cell has to ensure that its genome is duplicated once per cycle and segregated into the two daughter cells. The molecular mechanisms ensuring DNA replication once per cycle are evolutionarily conserved and based on modifications of the composition and phosphorylation state of protein complexes binding to specific DNA sequence motifs that define the chromosomal replication origins. Components of these origin recognition complexes (ORCs) are subject to CDK-mediated phosphorylation and APC-mediated degradation during specific cell cycle phases, ensuring that any replication origin can only be used in a time window between S phase entry and its own replication (reviewed by Musiałek and Rybaczek 2015). Chromosome segregation during a normal mitotic cell cycle requires attachment of its kinetochores to the mitotic spindle (Musacchio 2015; Pesenti et al. 2016) as well as separation of the two sister chromatids that are held together by cohesin protein

complexes, whose disassembly depends on selective proteolysis regulated by the APC (Yanagida 2005; Rankin and Dawson 2016).

Finally cytokinesis, possibly the evolutionarily most diverse cell cycle event, has to be coupled to the nuclear events of the cell cycle. Variability of the regulatory mechanisms involved reflects the structural diversity of cell division between fungi, mammals, and plants. In budding yeast, the earliest step toward cytokinesis (i.e., bud formation) is controlled by G1 phase-specific cyclins (Cvrčková and Nasmyth 1993), whereas the final separation of daughter cells depends on CDK deactivation and dephosphorylation of its targets by a specific phosphatase, encoded by *CDC14* (Kuilman et al. 2015). A similar pathway involving *CDC14* homologs also operates in fission yeast and mammals (Trautmann et al. 2004; Clifford et al. 2008). However, *bona fide CDC14* homologs are absent in higher plants (Kerk et al. 2008), and the post-mitotic stage of plant cytokinesis requires regulation of specific cytokinetic kinesins by a phosphorylation cascade involving an active CDK, rather than CDK inactivation (Sasabe and Machida 2014). This is yet another example of evolutionarily distant outgroups such as plants providing insights that allow identification of the necessary and sufficient molecular mechanisms of eukaryotic cell cycle control.

## 4.5 Variant Cell Cycles: Clues Toward Reconstructing Evolutionary History?

The sequence of cell cycle events may not be as invariant as the rather over-simplified summary outlined above (Sect. 4.1.) suggests. Meiosis, a process crucial for completion of the life cycle of sexual eukaryotes, can be viewed as a succession of two modified mitotic cell cycles. The first one replaces standard chromosome disjunction by pairing of homologous chromosomes and includes a modified anaphase without separation of sister chromatids, while the second one skips DNA replication. During the S phase in meiosis I of budding yeast, a meiosis-specific kinase related to but distinct from standard CDKs partially takes over the role of Cdc28 (Schindler and Winter 2006). In fission yeast and in plants, omission of the S phase in meiosis II depends on partial inhibition of cyclin degradation by meiosis-specific protein inhibitors (Peters 2005; Cromer et al. 2012). Pairing of homologous bivalents in the metaphase of *Arabidopsis* meiosis I requires a specialized CDK isoform (Zheng et al. 2014). Meiosis-specific cohesin isoforms ensure that sister chromatids remain connected during the first meiotic anaphase, and also contribute to bivalent pairing (Ding et al. 2016). Thus, meiosis not only reminds of a mitotic cell cycle on the phenotypic level, but also utilizes, in a modified context, much of the molecular apparatus controlling standard mitosis.

The regulatory apparatus of the mitotic cell cycle also participates in a process that does not strictly fit the formal definition of the cell cycle (see Sect. 4.1), namely endoreduplication of genomic DNA, producing cells with increased DNA contents.

This "endocycle," known, for example, from insect polytene chromosomes (see Frawley and Orr-Weaver 2015), is also commonly observed in differentiated plant tissues during vegetative development, where it can be brought about by changes in the expression levels of certain CDKs or CKIs (e.g., Boudolf et al. 2009; Wen et al. 2013). Thus, variations in gene expression levels and alternative use of paralogous genes can generate substantial deviations from the standard cell cycle scheme, resulting in whole genome amplification (as in the endocycle) or in reduction of genomic DNA content (as in meiosis).

These observations might provide some clues to explain a somewhat embarrassing evolutionary enigma. Even in its simplest theoretically analyzed form (see Sect. 4.4.2), cell cycle regulation is complicated, providing a nice example of the notorious irreducible complexity paradox (see Carreño et al. 2009). How could such a multicomponent molecular network have evolved, if omission of any of its parts jeopardizes the success of the crucial task, namely producing two identical cells out of one? A less-than-perfect controlling network would result in an error-prone mitotic cell cycle with several possible outcomes: (1) a rare success, that is, production of two daughter cells genetically identical to their mother; (2) an endocycle, or a failure of mitosis and/or cytokinesis, leading to polyploidy; (3) a whole genome non-disjunction, leading to one living polyploid cell and one dead enucleated cell; (4) a "reductive division," leading to halving of DNA content; or (5) mitotic failure resulting in aneuploidy. Although scenarios (4) and (5) would be fatal in cells with a single genome copy, they may be compatible with survival of at least one daughter if the mother cell underwent previous polyplodization. The remaining three possibilities are always guaranteed to produce at least one surviving cell. Thus, even an error-prone ancestral cell cycle might still, on average, have produced more than one live daughter per mother cell, enabling survival in the absence of more effective competitors, although the mean number of viable progeny would have been less than two, possibly substantially less.

Such an ancestral cell cycle would have continuously generated genetic variability (compare with the hypothesis of the last eukaryotic common ancestor as a population sharing a common thesaurus of genes; see Chapter 12 of this volume - Švorcová et al. 2018). It would have also produced a gradual increase rather than a decrease in the amount (and sequence content) of genomic DNA, because events resulting in gene gain are less likely to be fatal than those involving gene loss (at least assuming that only genes that contribute to fitness are kept in evolution; compare Thomas 1993). Although duplicated genes provide raw material for evolution, increasing the genome size may bring an increased metabolic burden from replication and promote accumulation of deleterious mutations (for a theoretical model see Markov and Kaznacheev 2016). Meiosis might thus have originally evolved as a repair mechanism to enable escape from this "polyploidy trap," as well as a means for repair of DNA damage by homologous recombination (Hurst and Nurse 1991; Wilkins and Holliday 2009). Once a mechanism of reductive nuclear division was established, it was followed by establishment of cellular and nuclear fusion mechanisms, an evolutionarily unique event that took place prior to the onset

of eukaryote diversification (Wilkins and Holliday 2009; Speijer et al. 2015) and paved the way toward evolution of eukaryotic sexuality.

## 4.6 Conclusions

The last two centuries have witnessed enormous progress in the field of cell biology, including research into the processes of cell multiplication. Since the second half of the twentieth century, most of the research interest has gradually shifted from the characterization of structural events of the cell cycle (where plant studies previously contributed key observations) toward a "cybernetic" perspective focusing on the molecular mechanisms responsible for timing and coordination of cell cycle events. Plants studies have lost some importance during this later stage, even though they contributed some important insights that enabled informed speculation on the evolutionary origins of the key mechanisms of cell cycle control. With the advent of new techniques, especially in vivo fluorescent labeling of proteins and advanced microscopy methods, focus is turning back to structural aspects of the cell cycle. However, these exciting new developments are beyond the scope of this review.

From the regulatory perspective, we now consider the eukaryotic mitotic cell cycle as a temporally conserved succession of events, controlled by a central oscillator comprising a set of CDKs, cyclins, and their regulators, with a specialized proteolytic machinery involving the APC playing a prominent part. Despite some variability in the number of paralogs of the key molecules and their functional diversification, this set is rather well conserved throughout evolution. The central oscillator regulates downstream events ranging from the nearly invariant (such as DNA replication) to evolutionarily diversified (such as cytokinesis). Even more diverse are the signaling pathways that modulate the function of the central oscillator. Meiosis and endoreduplication, processes that lead to controlled changes in the genomic contents of the cell, utilize parts of the molecular apparatus of the standard mitotic cell cycle, providing clues toward reconstructing an evolutionary scenario that may have produced the precise cell cycle control known from extant eukaryotes.

**Acknowledgements** I dedicate this essay to the memory of Zdeněk Neubauer (1942–2016), who taught me, among many other things, that stories make the difference between history and historiography. I thank the Ministry of Education, Youth and Sports of the Czech Republic for generous financial support from the NPUI LO 14017 project, and teams of The Biodiversity Heritage Library (www.biodiversitylibrary.org), Internet Archive (www.archive.org), Münchener Digitalisierungszentrum (www.digitale-sammlungen.de), and Project Gutenberg (www.gutenberg.org) for making the historical literature available on-line.

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# Chapter 5
# Plant Microtubule Research: A Short History


**Kateřina Schwarzerová**

**Abstract** Microtubules are hollow tubes composed of tubulin subunits. These ubiquitous structures are found in all eukaryotic cells. This short review describes the most important moments of plant microtubule research, which was important for understanding the microtubular cytoskeleton in eukaryotic cells. Cytoplasmic microtubules were first described in plant cells. Similarly, the structure of eukaryotic flagellum consisting of microtubules was first studied in plant spermatic cells. Today, integration of the knowledge from various fields and organisms is proving beneficial in advancing microtubular cytoskeleton research.

## 5.1 Introduction

In 2013, 50 years had passed from the first description of tiny cytoplasmic tubules, called microtubules (Ledbetter and Porter 1963). This anniversary was commemorated by several journals, which paid tribute to microtubule research. A special issue of *The Plant Journal* ("A glorious half-century of microtubules," volume 2, issue 2, 2013) included a series of articles summarizing our current state of knowledge of plant microtubule structure and function. A retrospective contribution in the same special issue contains the personal recollections of leading scientists who witnessed and shaped plant microtubule research (Hepler et al. 2013). This study represents a very precious glimpse into the exciting era of early studies of microtubules for those who entered the field later, when immunofluorescence, GFP technology, and in vitro assays were considered a matter of course rather than brand new achievements. The history of 60 years of cytoskeleton research, initiated with the actomyosin discovery, is summarized in a special *Nature* supplement ("Nature Milestones in Cytoskeleton," 2008). I also refer readers to a publication commemorating the anniversary of 50 years of tubulin discovery (Borisy et al. 2016), where six leaders of microtubule research discuss the greatest achievements

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in the field. The current review attempts to highlight the contribution of plant microtubule research to the general knowledge of microtubular function in eukaryotic cells.

## 5.2 Eukaryotic Flagellum

Microtubules are hollow tubes with an outer diameter of 25 nm, which is too tiny to be observed using a standard light microscope. However, microtubules also form more robust structures in the cytoplasm, whose presence was noted by early microscopists. Examples of these are flagella and mitotic spindles.

Eukaryotic flagella were probably the first structures observed to contain fibrils, which were later described as microtubules (for a short history of early flagellum observations, see Hepler et al. 2013). Irene Manton, a British botanist, used UV microscopy to observe disintegration of moss spermatozoid flagellum into several threads. The disintegration occurred only under a specific fixation protocol and staining conditions. Manton was even able to recognize that the total number of filaments forming the flagellum was 11. Equipped with a new electron microscope, whose era in biology was just beginning, Manton returned to this observation. The study of moss flagellum with electron microscopy enabled Manton and Clarke (1952) to deduce the 9 + 2 model of the axoneme (Fig. 5.1; Manton and Clarke 1952). Their work thus represents the first description of the eukaryotic flagellum structure, which was strikingly precise compared with the current model of flagellum. The universality of the model was later confirmed in other organisms such as brown algae (Manton et al. 1953).

## 5.3 Microtubules

Colchicine played an important role in the discovery of microtubules. This molecule, isolated from *Colchicum autumnale*, was long known as a specific disruptor of mitotic spindles. In 1962, Paul Green described the effect of colchicine on *Nitela*

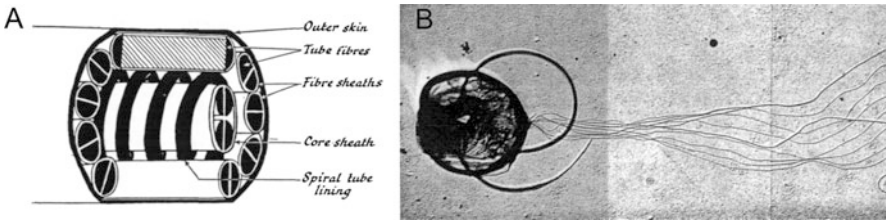


**Fig. 5.1** The first model of a flagellum (**a**), which was deduced on the basis of a study of *Sphagnum* flagella (**b**). Reproduced with permission from Manton and Clarke (1952)

internode cells. In response to colchicine treatment, plant cells became round and lost their original shape (Green 1962). Green assumed that colchicine-sensitive filaments of mitotic spindles were present at the plant cell periphery, where they controlled the cell wall assembly. He therefore predicted the existence of cortical microtubules without seeing them. Single cytoplasmic microtubules were observed later with the use of electron microscopy and the availability of a new method of fixation using glutaraldehyde (Sabatini et al. 1963).

Osmium fixation, used for preparation of cells observed using electron microscopy, supplemented with a glutaraldehyde prefixation step, was a great methodological advance because this procedure beautifully preserved cytoplasmic structures. Using the new fixation procedure, Ledbetter and Porter (1963) could show microscopic tubular structures found in the cytoplasm of several plant cells. In the same year, Slautterback (1963) demonstrated microtubules in animal cells. Whereas Slautterback considered microtubules to be membranous structures, Ledbetter and Porter recognized their ubiquitous distribution in the cytoplasm and noted their structural resemblance to filaments of the mitotic spindle and filaments forming the flagellum. In a subsequent paper, they described, for the first time, that the wall of microtubules is formed by 13 subunits (Fig. 5.2; Ledbetter and Porter 1964). Their seminal paper gives the first description of microtubules in eukaryotic cells and also showed that microtubules are parallel to cellulose in the primary cell wall (Ledbetter and Porter 1963). An observation of Hepler and Newcomb (1964) that microtubules are located just beneath cell wall ingrowths in newly formed tracheary elements in Coleus suggested that secondary cell wall deposition is assisted by microtubules as well. Therefore, the observations of Green (1962), Ledbetter and Porter (1963), and Hepler and Newcomb (1964) were crucial for establishing the hypothesis that cortical microtubules control the deposition of cellulose in the cell wall. Interestingly, the final evidence for this hypothesis was provided more than 40 years later, when fluorescently tagged cellulose synthases were shown to follow trajectories oriented along cortical microtubules in living cells (Paredez et al. 2006). Many aspects of both the control of cortical microtubule orientation and their role in cell wall synthesis are still under investigation.

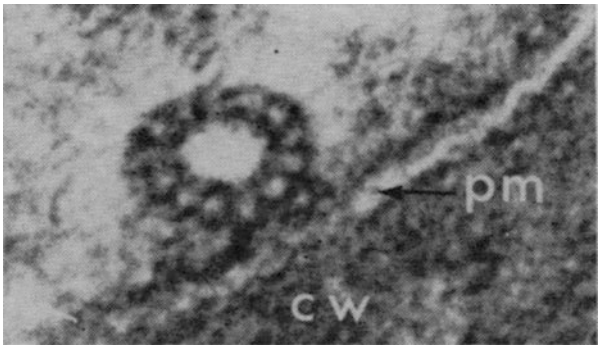


**Fig. 5.2** The first description of a microtubule structure. Transverse section of a microtubule from the cortex of *Juniperus chinensis* root tip cell (*pm* plasma membrane, *cw* cell wall). Electron micrograph, 740,000×. Reproduced with permission from Ledbetter and Porter (1964)

## 5.4 Mitotic Spindles and Tubulin Discovery

Similar to flagella, the mitotic spindle is a prominent structure visible under a standard light microscope, and thus known to biologists long before the discovery of microtubules. The filamentous character of mitotic spindles could be observed using advanced polarized light microscopy in living cells because of their birefringence (Inoue and Bajer 1961). In vivo observations of mitotic spindle dynamics using polarized light microscopy and the discovery of microtubules using electron microscopy resulted in the construction of the first model of animal and plant mitotic spindle structure, including premitotic spindle and phragmoplast, formed by dynamic microtubules capable of depolymerization and polymerization (Inoué and Sato 1967): "Spindle fibers in living cells are labile dynamic structures whose constituent filaments (microtubules) undergo cyclic breakdown and reformation" (Inoué and Sato 1967).

Microtubules were thus thought to be responsible for chromosomes movement in mitotic spindles. However, the protein that formed microtubules was not yet known. Tubulin was identified thanks to its affinity to colchicine. Colchicine, as mentioned before, specifically destroys mitotic spindles as well as cytoplasmic microtubules. A protein that bound to colchicine and was highly enriched in dividing cells was identified as a subunit of microtubules (Borisy and Taylor 1967a). The mechanism of colchicine action was recognized as involving binding to microtubular subunits, thus inhibiting their ability to polymerize (Borisy and Taylor 1967b): "A plausible explanation of the mechanism of [colchicine] action is provided by assuming that binding of colchicine prevents assembly of the subunit into a microtubule" (Borisy and Taylor 1967b).

## 5.5 Immunofluorescence Microscopy

Electron microscopy enabled biologists to identify microtubules as ubiquitous cytoplasmic structures in all eukaryotic cells. Tubulin isolation was an important prerequisite for the production of specific antibodies. With the availability of an antibody recognizing tubulin, immunofluorescence microscopy was another important methodological achievement in the field. In plant cells, the application of antibodies was limited by the existence of the cell wall, which was not penetrable by such large molecules. Therefore, cell wall digestion by enzymes was needed for the delivery of antibodies into the plant cytoplasm. The first observation of immunofluorescently labeled plant microtubules was performed by Clive Lloyd in a suspension of cells using an antibody against bovine brain tubulin (Lloyd et al. 1979). Immunofluorescence techniques thus provided the opportunity to study the organization of microtubules at the level of the whole cell and tissue (Wick et al. 1981), using either wide-field fluorescence or confocal microscopy.

## 5.6 Dynamic Structures

Microtubules are dynamic structures. Based on early observations of mitotic spindles using polarized light microscopy in living cells, it was obvious that microtubules can shrink and elongate. Electron microscopy enabled researchers to identify microtubules and to study their structure. Immunofluorescence enabled study of microtubules at the tissue level, as well as changes in their organization during the cell cycle. However, both methods produced static pictures, which do not reflect the actual dynamics of microtubules. The use of polarized light microscopy, suitable for dynamic studies in non-plant cells, was limited in plant cell studies because of the strong birefringence of cellulose in cell walls. A new approach involved covalent binding of fluorescent molecules to proteins, referred to as cytochemistry. Introduction of fluorescently labeled protein into the cytoplasm resulted in the first observation of the dynamics of single microtubules in fibroblasts (Sammak and Borisy 1988), and soon also in plant cells. Microinjection of covalently labeled bovine tubulin resulted in bovine tubulin incorporation into plant microtubular arrays so that their changes during the transition through the mitosis could be followed in vivo (Zhang et al. 1990). Covalently modified tubulin microinjection was also used for the first visualization of cortical microtubule dynamics (Wasteneys et al. 1993). Thus, just before the dawn of the green fluorescent protein (GFP) age, cytochemical methods were highly instructive in imaging of plant microtubular dynamics, and also for demonstrating the conserved structure and role of tubulin when bovine tubulin copolymerized with plant tubulin.

## 5.7 Dispersed Microtubule Organizing Centers

Plant cell research has been very instructive for the understanding of microtubule organizing centers (MTOCs; Pickett-Heaps 1971) as flexible structures, as suggested by Mazia (1984). Early studies suggested that higher plants lack centrosomes with two centrioles, which organize microtubules in most animal cells, or the spindle pole bodies of fungal cells (for review of various MTOCs, see Yubuki and Leander 2013). Although primitive higher plants with motile sperm form specialized structures called bicentrioles or blepharoplasts, which give rise to centrioles that organize the flagellar apparatus of the sperm cell (Hepler et al. 2013), higher plants do not form distinct centrosome-like MTOCs. Nevertheless, plant cells are perfectly capable of organizing ordered microtubular arrays during interphase and bipolar spindles during mitosis. The discovery of gamma-tubulin, the third member of the tubulin family, and its description as a universal nucleator of microtubules, furthered understanding of MTOCs in higher plants. Gamma-tubulin was demonstrated to be present in plants, where it localized to the minus ends of microtubules (Liu et al. 1994). The localization of gamma-tubulin and other proteins organizing microtubules led to the hypothesis of dispersed MTOCs in plants (Wasteneys

2002). A recent hypothesis is that the existence of plant cell walls and the loss of flagellated sperm are probably linked with the evolution of specific mechanisms in plants for the control of microtubular organization that do not rely on a distinct MTOC. For example, the fascinating self-organization properties of cortical microtubules started to be understood better in 2005 with the description that new microtubules are nucleated as branches on the extant cortical microtubules from multiple gamma-tubulin microtubule-associated centers (Murata et al. 2005). Current research has confirmed that, in higher plants, the MTOC is partially transformed into a protein network operating at the cell cortex, controlling the polarity of plant cell division (Schaefer et al. 2017).

## 5.8 Conclusions and Prospects

Research on plant cells is credited for constructing the first model of axoneme structure and the first identification of microtubules as ubiquitous cytoplasmic structures. Tubulin has proved to be a highly conserved protein. Thanks to this very important fact, plant research benefited from experiments with animal tubulin. Although brain tissue, by far the best source of pure tubulin, is absent in plants, brain tubulin copolymerization with plant microtubular arrays became an effective marker tool in plants. Similarly, animal anti-tubulin antibodies usually show good cross-reactivity with plant tubulins. Since GFP technology and genome sequencing programs accelerated progress in biology, it is important to continue integrating knowledge from various fields. Indeed, some discoveries carried out on plant material have shaped future research of eukaryotic cells. For example, most higher plants do not form the flagellum in any stage of life. However, lower plants (green algae) possess flagella, and these plant cells are credited for many advances in the study of the eukaryotic flagellum in contemporary research. Green unicellular algae such as *Chlamydomonas* have two motile flagella. *Chlamydomonas* became a model organism for flagellum structure and function studies because of its simple life cycle, synchronized growth, and availability of methods for biochemical and genetic studies (for a review, see Harris 2001; Dutcher 2014). Early studies of *Chlamydomonas* led to detailed characterization of proteins involved in axoneme assembly and function, such as dyneins (DiBella and King 2001), or of proteins needed for intraflagellar transport (Taschner and Lorentzen 2016), a process that was first described in *Chlamydomonas* (Kozminski et al. 1993). In 2000, Pazour et al. found that a homolog of the *IFT88* gene, which is involved in axoneme assembly in *Chlamydomonas*, is mutated in mice with polycystic kidney disease (Pazour et al. 2000). This discovery led to the identification of primary cilia, previously considered vestigial, as an important sensing organelle in mammalian cells (Berbari et al. 2009), whose dysfunction results in several human pathologies (Fliegauf et al. 2007; Pan et al. 2005).

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# Chapter 6
# Plant Actin Cytoskeleton: New Functions from Old Scaffold


**Ana Paez-Garcia, J. Alan Sparks, Louise de Bang, and Elison B. Blancaflor**

**Abstract** The actin cytoskeleton plays an essential role in several biological processes in plants, including cell division, cell expansion, organelle movement, vesicle trafficking, and the establishment of polar cell growth. To function properly, actin has to undergo continuous rounds of dynamic remodeling as the plant is presented with a constant stream of endogenous and exogenous signals. Remodeling of the actin cytoskeleton in plants is modulated by a multitude of highly conserved actin-binding proteins (ABPs). In recent years, additional proteins that interact directly or indirectly with actin have been uncovered. Although the precise roles of these newly described proteins have yet to be fully understood, initial studies suggest that they could confer actin functionalities and remodeling mechanisms that are distinct from those found in other eukaryotes. In this chapter, we briefly highlight some of the recent advances toward understanding how the actin cytoskeleton modulates plant growth, form, and adaptation to the environment. We focus primarily on live cell actin tools and on new insights about plant actin and ABP function culminating from the use of such tools. We also discuss some recently discovered plant proteins that function in actin-mediated biological processes that are unique to plants.

## 6.1 Introduction

The networks of filamentous protein polymers that make up the cell skeleton (cytoskeleton) regulate a multitude of intracellular processes essential for life. Like other eukaryotic cells, plant cells rely on the cytoskeleton to power the movement of organelles and to serve as tracks for vesicles to reach their correct

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destination within the cell (Staiger 2000; Hussey et al. 2006; Wada 2013; Fu 2015; Geitmann and Nebenführ 2015; Wang et al. 2017b). Cargo carried by these vesicles includes polysaccharide precursors for assembly of the rigid cell wall that supports plant growth and proteins destined for the plasma membrane (PM) and other endomembrane compartments (Rounds et al. 2014; Li et al. 2015a). Microtubules and filamentous actin (F-actin) are the major polymers that constitute the cytoskeleton. The latter component, which is the focus of this chapter, assembles from a pool of globular actin (G-actin) monomers to form 5–7 nm two-handed helical structures (Li et al. 2015a). When viewed at the resolution of a light microscope, fluorescently tagged F-actin in diverse plant cells appears as dense networks of thick cables and fine filaments (Fig. 6.1a). For plant cells to grow normally and eventually attain their final shapes within the plant body, this elaborate F-actin network has to undergo continuous rounds of dynamic remodeling (Fig. 6.1b; see Sect. 6.2.3). How cellular F-actin is reorganized at the global and local scales, to enable plants to readjust their developmental programs so that they can adapt to their constantly changing environment, has been the subject of intense research (Smertenko et al. 2010; Day et al. 2011; Pleskot et al. 2013; Henty-Ridilla et al. 2013; Li et al. 2014, 2015a).

The process by which the higher order structure of the actin cytoskeleton is remodeled is under tight regulation by a plethora of actin-binding proteins (ABPs). Among the known ABPs in animal and fungal cells, about 150 have homologs in plants (Meagher and Fechheimer 2003). These include the monomer-binding actin depolymerizing factors (ADFs) and profilins (Sun et al. 2013; Inada 2017), and proteins such as formins, fimbrins, and villins that nucleate, bundle, and crosslink F-actin (Blanchoin and Staiger 2010; Thomas 2012; Huang et al. 2015). Actin nucleating factors such as those belonging to the actin-related protein (ARP)2/3-WAVE/SCAR complex are also widespread in plants and reported to be involved in organ growth and the response to abiotic stresses (Dyachok et al. 2008, 2011; Zhao et al. 2013; Facette et al. 2015; Zhou et al. 2016). Moreover, like other eukaryotes, plants power the movement of their organelles along F-actin through myosin-motors. In plants, the class XI myosins are homologous to the fungal and animal class V myosins (Madison and Nebenfuhr 2013; Ueda et al. 2015). There is recent evidence that plant myosins not only serve as motors to drive organelle movement, but also contribute significantly to overall F-actin organization and structure (Peremyslov et al. 2010).

ABPs have also been proposed to regulate F-actin-dependent crosstalk between adjacent cells. This F-actin-dependent communication occurs at cross-walls of cells in different plant organs and is mediated by endocytosis, vesicular transport, and recycling activities. Cross-walls are actin-enriched domains in which two types of actin arrays can be found. One dense network of short filaments is located close to the PM and is involved in vesicle recycling and endocytosis. Additionally, a network of thick and long filaments runs across the cell longitudinally and interconnects opposite cross-walls (Němec 1901). Both these arrays are essential for cell-to-cell communication. Signaling in plants requires transport of substances through two barriers: the PM and the cell wall. To fulfill this requirement, it is

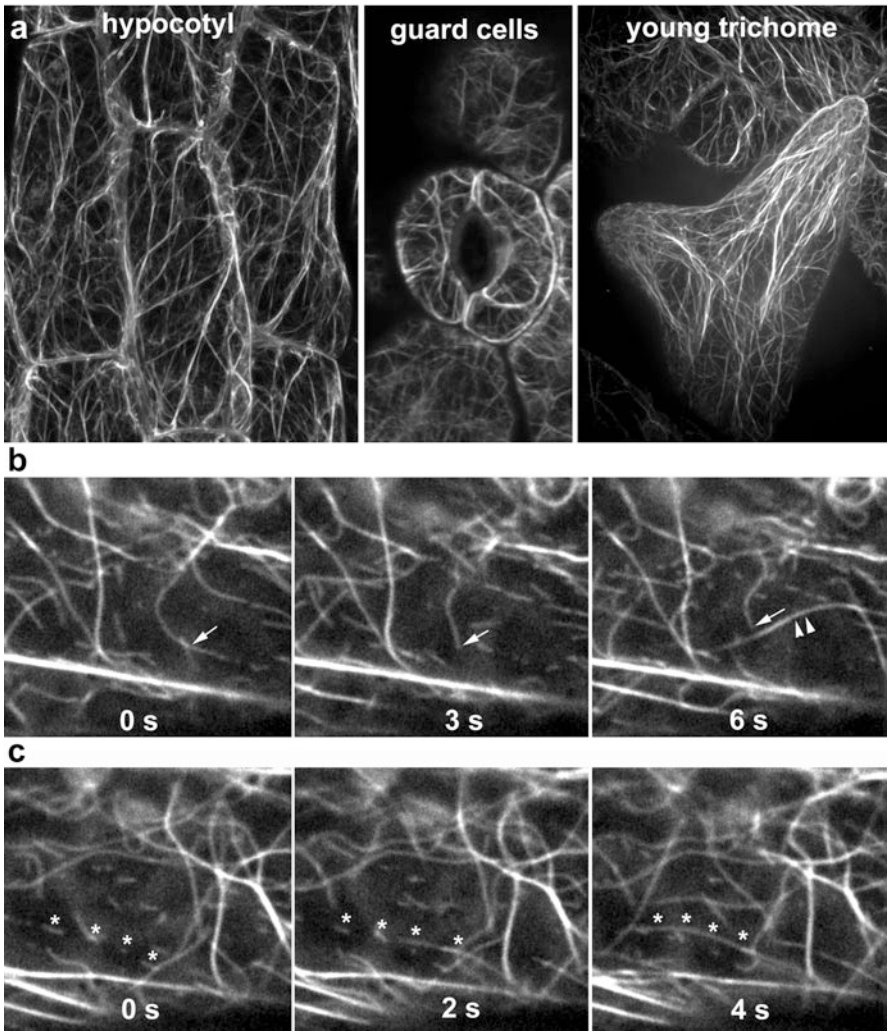


**Fig. 6.1** Actin organization and remodeling in living cells of *Arabidopsis thaliana* expressing genetically encoded F-actin-binding fluorescent reporters. (**a**) The actin cytoskeleton in various plant cell types consists of thick cables and a fine network of filaments. (**b**, **c**) Cortical actin stochastic dynamics in epidermal cells of dark-grown hypocotyls. Time-lapse spinning-disc confocal microscopy shows various types of individual actin filament dynamics. Severing (arrows in **b**), rapid elongation (double arrowheads in **b**), and small F-actin fragments combining into one longer filament (asterisks in **c**)

essential that functional links between the cytoskeleton, cell wall, and PM are formed. ABPs with transmembrane domains as well as domains that direct them to the cell wall are good candidates for carrying out functions at cross-walls. Formins, myosins, and profilins have been localized at the cross-walls, suggesting

that these ABPs participate in F-actin dynamic remodeling and transport of molecules across cellular boundaries (Baluška and Hlavačka 2005). More specifically, group I formins are thought to be important for the formation of dense actin meshworks, whereas group II formins seem to be relevant for the organization of thick actin bundles (Deeks et al. 2005).

For the benefit of the reader, a summary of plant ABP homologs and their reported functions in basic plant physiological processes are presented in Table 6.1. However, because these plant ABPs have been covered in several reviews (Higaki et al. 2007; Thomas 2012; Henty-Ridilla et al. 2013; Cvrckova et al. 2014; Wang and Hussey 2015; Komis et al. 2015), they are not discussed extensively here. We highlight conserved ABPs in which recent breakthroughs about their mode of action in remodeling the plant actin cytoskeleton have been described. Furthermore, we touch on recently described plant ABPs and other proteins that have been linked directly or indirectly to actin; detailed study of these proteins should bring better understanding of actin-dependent biological processes that are unique to plants. We also review some of the live cell imaging tools that have helped advance our understanding of actin function in plants.

## 6.2 Live Cell Methods for Studying Actin in Plants

Studying actin in plants requires the implementation of methods to image its organization in the cell. This section reviews live cell actin probes developed over the years that have enabled rapid advances in understanding the plant actin cytoskeleton. Early studies of actin organization in plants were typically conducted on chemically fixed or rapidly frozen tissue using phalloidin, a toxin from the mushroom *Amanita phalloides* (Miyamoto et al. 1986; Wendel and Dancker 1987; Waller and Nick 1997) or actin-specific antibodies via indirect immunofluorescence microscopy (Lovy-Wheeler et al. 2005; Dyachok et al. 2016). Phalloidin binds to F-actin and can be tagged with a fluorophore, enabling F-actin networks to be visualized by fluorescence microscopy (Blancaflor and Hasenstein 2000). Although such methods have led to new discoveries about actin organization in plant cells, such as the cortical actin fringe in pollen tubes (Lovy-Wheeler et al. 2005), their popularity has dwindled in the past decade because of the convenience of creating live cell genetically encoded F-actin probes. Prior to the advent of genetically encoded fluorescent biomarkers, microinjection of fluorescently tagged phalloidin was used to visualize actin in living plant cells (Schmit and Lambert 1990; Valster et al. 1997). However, in addition to being technically demanding, microinjection of living plant cells has a low success rate and can be prone to artifacts. Whereas the methods noted above for imaging actin require careful optimization of fixation, arduous sample processing steps (e.g., sectioning) (Wu et al. 2012; Avci and Nakashima 2015), and in some cases specialized equipment (Valster et al. 1997; Lovy-Wheeler et al. 2005), the generation of genetically encoded reporters requires only an initial investment in molecular

**Table 6.1** Plant actin-binding proteins with conserved homologs in other eukaryotes

| Actin-binding protein | Biochemical functions | Plant biological functions | References |
|---|---|---|---|
| Profilin | Inhibits actin polymerization and promotes F-actin elongation<br>[a]Depolymerization and monomer recycling | Cell elongation, pollen tube growth | Sun et al. (2013)<br>Fan et al. (2013) |
| Formin | Promotes nucleation and filament elongation<br>[c]Crosslinking activity | Root hair and pollen tube growth<br>Cell division and root development | Wang and Hussey (2015)<br>Xue et al. (2011)<br>Deeks et al. (2005) |
| ARP2/3 complex | Promotes filament branching, nucleation | Root growth<br>Stomatal opening<br>Shaping and elongation of epidermal cells, trichome development<br>Plant cell development and morphogenesis<br>Mitochondrial-dependent $Ca^{2+}$ signaling in response to salt stress,<br>Regulator of autophagy during abiotic stress | Dyachok et al. (2008, 2011)<br>Li et al. (2013)<br>Havelkova et al. (2015), Zhou et al. (2016)<br>Facette et al. (2015)<br>Zhao et al. (2013)<br>Wang et al. (2016) |
| Capping protein (CP) | [d]Capping activity | Favors thermotolerance in *Arabidopsis* plants coping with heat stress<br>Hypocotyl elongation<br>Plant innate immunity | Wang et al. (2012)<br>Li et al. (2012, 2014, 2015b, 2017a) |
| Villin | [a]Depolymerization and monomer recycling<br>[b]Severing activity<br>[c]Crosslinking activity<br>[d]Capping activity | Pollen tube growth | Huang et al. (2015) |
| Fimbrin | [c]Crosslinking activity | Pollen germination and pollen tube growth | Wu et al. (2010), Su et al. (2012) |
| ADF/cofilin | Promotes nucleation of G-actin<br>[a]Depolymerization and monomer recycling<br>[b]Severing activity<br>[c]Crosslinking activity | Controls cell elongation, organ morphology and flowering time<br>Plant response to abiotic stresses including drought, salinity, and both high and low temperature<br>Plant response to biotic stress | Henty et al. (2011), Inada (2017), Zhu et al. (2017)<br>Fu et al. (2014), Henty-Ridilla et al. (2014) |
| AIP1 | Enhances the severing and nucleation promoting activities of ADF/Cofilin<br>Assists CP in its capping activity | Affects tip growth and modulates planar polarity | Ketelaar et al. (2004a, 2007), Kiefer et al. (2015) |

[a]Depolymerization and monomer recycling: Returns G-actin to the monomer pool by rearranging actin filaments that have been previously severed at the pointed end
[b]Severing activity: Cuts and disassembles F-actin
[c]Crosslinking proteins that assemble F-actin into networks and bundles
[d]Capping activity: Blocks F-actin barbed ends after filament severing, preventing its polymerization

cloning and plant transformation. Plasmid and/or seed from transgenic lines (e.g., *Arabidopsis thaliana*) expressing these genetically encoded reporters can be easily shared with other researchers. Since they were first introduced in plants, these genetically encoded fluorescent protein-based F-actin reporters have been used extensively for study of actin dynamics and the effects of gene mutations/environmental perturbations on F-actin organization in living plant cells. A selection of studies in which these genetically encoded F-actin probes have been used for plant biological research is summarized below.

### 6.2.1 *Live Cell Imaging Approaches for Studying Actin and Caveats Associated with Their Use*

The first generation of genetically encoded fluorescent probes for visualizing F-actin in living plant cells was reported by Kost et al. (1998). The authors used the F-actin binding domain of mouse (*Mus musculus*) Talin protein (mTalin) fused to green fluorescent protein (GFP). Expression of the GFP-mTalin construct revealed extensive networks of F-actin in various cell types (Kost et al. 1998). Although adverse effects on plant growth and actin dynamics were eventually reported with certain versions of these GFP-Talin constructs (Ketelaar et al. 2004b; Wang et al. 2004; Holweg 2007; Dyachok et al. 2014), they continue to be widely used by the plant scientific community.

After the Talin-based F-actin probes, a number of laboratories introduced another set of live cell F-actin markers in plants. Most notable were probes based on the second actin-binding domain (fABD2) of the F-actin crosslinking protein, fimbrin (Sheahan et al. 2004; Wang et al. 2004; Voigt et al. 2005; Sano et al. 2005; Guan et al. 2014). These fimbrin-GFP fusions were shown to decorate a finer network of F-actin compared with Talin-based probes in certain plant cell types (Sheahan et al. 2004; Wang et al. 2004; Voigt et al. 2005). An improvement in signal-to-noise ratio for fABD2-based reporters was accomplished by adding single fluorescent proteins to both the C- and N-termini of fABD2 (Fig. 6.2) (Wang et al. 2008; Dyachok et al. 2014). However, like Talin-based reporters, some versions of these fABD2-based probes were reported to cause plant growth defects (Wang et al. 2008; Dyachok et al. 2014), a probable consequence of high levels of expression of the fusion protein and its negative impact on actin dynamics (van der Honing et al. 2011; Montes-Rodriguez and Kost 2017). Nonetheless, like Talin-based probes, fABD2-based probes have been widely adopted by the plant scientific community for study of plant actin function.

Another genetically encoded F-actin reporter that has gained popularity for live cell imaging of actin in plants is one that is based on a 17 amino acid peptide from the budding yeast *Saccharomyces cerevisiae* ABP140 called Lifeact (Riedl et al. 2008). Following the first reports of Lifeact-GFP as a versatile probe for F-actin in animals and yeast, a number of laboratories rapidly implemented its use in plants.

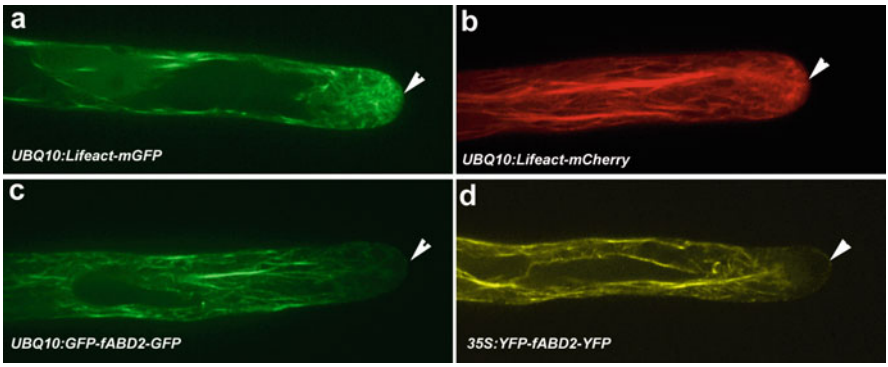


**Fig. 6.2** Actin organization in elongating *Arabidopsis thaliana* root hairs. (**a**, **b**) Two versions of the Lifeact reporter clearly mark a meshwork of actin filaments at the apical-most dome of the root hair (arrowheads). (**c**, **d**) fABD2 reporters appear to have lower affinity for actin filaments at the apical-most dome of the root hair (arrowheads)

Various versions of Lifeact-based fluorescent reporters were first used to visualize F-actin in *A. thaliana*, *Nicotiana tabacum* Bright-Yellow 2 (BY-2) suspension cells, the liverwort *Marchantia polymopha*, the moss *Physcomitrella patens*, and pollen tubes of *N. tabacum* and *Lilium formosanum* (Era et al. 2009; Vidali et al. 2009; Smertenko et al. 2010). Many studies have now used these Lifeact-based probes after their first introduction in plants.

Talin, fABD2, and Lifeact fluorescent protein fusions are the most popular reporters used to study actin in living plants. Although not as widely used as the Talin-, fABD2- and Lifeact-based reporters, other genetically encoded F-actin reporters, including GFP-ADF1 and LIM2b-GFP, are just as versatile, particularly for visualizing actin in growing pollen tubes (Chen et al. 2002; Wilsen et al. 2006; Cheung et al. 2008).

Because these F-actin-binding fluorescent fusions can be detrimental to plant growth when expressed at high levels (Vidali et al. 2009; Montes-Rodriguez and Kost 2017), the use of alternative promoters such as *UBIQUITIN10* (*UBQ10*) and *ACTIN3* to drive fusion protein expression has also been attempted (Dyachok et al. 2014; Jasik et al. 2016). In some cases, transgene silencing was minimized when weaker promoters were used to drive expression of the fusion proteins (Dyachok et al. 2014).

Recently, a method that relies on antibodies from the serum of camels (*Camelus dromedarius*) was explored as an alternative to F-actin reporters utilizing fluorescently tagged actin-binding domains/peptides. Some antibodies from Camelids are devoid of light chains but are still capable of strong antigen binding (Hamers-Casterman et al. 1993). The small molecular mass of these heavy chain antibodies or nanobodies makes them ideal biomarkers, particularly the so-called chromobodies, which consist of the binding moiety of nanobodies fused with a fluorescent protein (Rothbauer et al. 2006). Transient expression of a commercially available yellow fluorescent protein-tagged actin-chromobody (YFP-actin-Cb) in

tobacco leaves revealed structures corresponding to F-actin, adding yet another tool for visualizing actin networks in plants (Rocchetti et al. 2014; Wang and Hussey 2017).

The use of F-actin live cell reporters has enabled a number of fundamental insights into the higher order organization and dynamics of actin in plants. For example, dynamic actin focal points and patches were uncovered in tips of moss protonema (Vidali et al. 2009). Moreover, the presence of a cortical actin fringe, first demonstrated in high-pressure frozen pollen (Lovy-Wheeler et al. 2005), was verified using reporters based on Lifeact, Talin, and ADF (Wilsen et al. 2006; Vidali et al. 2009; Cheung et al. 2008; Montes-Rodriguez and Kost 2017). It was recently shown through live cell imaging of Lifeact-GFP that the pollen cortical actin fringe facilitates tip-directed movement of vesicles (Qu et al. 2017). Furthermore, villins and formin 1 were involved in the assembly and maintenance of the cortical actin fringe (Li et al. 2017b; Qu et al. 2017). These genetically encoded F-actin probes also enabled quantification of the dynamics and behavior of individual actin filaments in the cortex of etiolated *Arabidopsis* hypocotyls (Fig. 6.1b). Using a technique called variable angle epifluorescence microscopy (VAEM), individual actin filaments that make up the cortical actin array were shown to undergo complex dynamic remodeling consisting of buckling, straightening, severing, and rapid growth (Staiger et al. 2009). Another study uncovered dynamic actin ring-like structures called acquosomes and an additional mechanism for cortical actin remodeling that involved short actin filaments combining into a longer filaments (Smertenko et al. 2010). The ability to quantify the intricate dynamics of individual actin filaments has paved the way for new discoveries on the mode of action of some plant ABPs (see Sect. 6.2.3).

Although, genetically encoded fluorescent protein F-actin reporters have enabled rapid advances in understanding plant actin function, we cannot discount the fact that each reporter has advantages and disadvantages (Du and Ren 2011). On the basis of studies comparing the performances of these different live cell F-actin probes, it is clear that one has to take into account the expression levels of the fusion proteins, an issue that can be addressed in part by the choice of promoter (Dyachok et al. 2014; Jasik et al. 2016; Montes-Rodriguez and Kost 2017). Furthermore, certain live cell F-actin probes appear to be more suitable for studies of tip-growing cells (e.g., Cheung et al. 2008; Vidali et al. 2009; Sparks et al. 2016; Montes-Rodriguez and Kost 2017; Fig. 6.2). In this regard, a Lifeact-YFP fusion enabled the visualization of F-actin dynamics in polarizing *Arabidopsis* zygotes. Using multiphoton microscopy, F-actin was found to form an apical cap and longitudinal cables along the apical–basal axis of the zygote that was reminiscent of tip-growing cells (Kimata et al. 2016).

The quality of F-actin labeling can also be influenced by the type of fluorophore tag, the length of the linker between the fluorophore and F-actin binding domain, and whether the fluorescent protein tag is placed at the C- or N-terminus of the F-actin binding domain (Vidali et al. 2009; Dyachok et al. 2014; Cvrckova and Oulehlova 2017; Montes-Rodriguez and Kost 2017). Mechanisms explaining differences in F-actin labeling patterns and performance for the various live cell actin

probes are not clear. To be confident of the results obtained with the live cell F-actin reporters, each probe has to be critically evaluated and compared with optimally fixed tissue or phalloidin-stained samples (Wilsen et al. 2006; Vidali et al. 2009; Dyachok et al. 2014).

### 6.2.2 *Quantification of Actin Organization in Plant Cells*

Although the use of live cell probes for visualizing actin in plants has increased dramatically in the past decade, elucidation of plant actin function would not have advanced if not for parallel work on developing computer software aimed at extracting quantitative information from microscopy data sets. This subsection briefly describes some of the tools developed to quantify actin organization and dynamics. Some of these tools are summarized in Table 6.2.

The most common metrics used by plant biologists for quantifying actin organization are those introduced by Higaki et al. (2010). In their image analysis

**Table 6.2** Tools for quantitative analysis of actin organization and dynamics

| Tool/software | Actin-related parameters measured by the tool | Reference |
|---|---|---|
| Cluster analysis and quantification | Actin organization: mean angular difference (as an indicator of cytoskeletal orientation), skewness (bundling), and occupancy (density) | Higaki et al. (2010) |
| Microfilament analyzer | Actin organization: identify and quantify F-actin orientation (increment of degrees of orientation from the horizontal position) | Jacques et al. (2013a, b) |
| Shape analysis software | Actin organization: thickness, multi-orientation index, complexity, and binarized pattern | Kimori et al. (2016) |
| Fast Fourier transform (FFT) | Actin organization: eccentricity | Vidali et al. (2010), Burkart et al. (2015) |
| Correlation coefficient analysis | Global actin dynamics: changes in the intensity of the fluorescence signal at all pixel locations between time points | Vidali et al. (2010), Burkart et al. (2015) |
| Quantitative analysis of cytoskeletal kymograms (QuACK) | Actin dynamics: dynamic filament end turn-over and lateral mobility | Cvrckova and Oulehlova (2017) |
| Quantification of system-wide dynamics | Actin network properties to compare arrangements across conditions and time to predict organelle flow and assess cargo transport efficiency (thickness, bundling, alignment, reachability, robustness, etc.) Golgi network properties (number, direction, velocity, and combinations of these parameters) | Breuer et al. (2017) |

framework, they used semi-automated clustering of cytoskeletal structures from microscopy images derived from lines expressing GFP-fABD2. Global actin reorganization events, particularly bundling (i.e., skewness) and density (i.e., occupancy) that accompany stomatal movement, were collected in *Arabidopsis* plants expressing GFP-fABD2. These image analysis methods showed that the extent of F-actin bundling in guard cells plays a prominent role in stomatal movements (Higaki et al. 2010). Analyzing skewness in grapevine expressing the GFP-fABD2 construct revealed that actin in guard cells responds to pathogen attack and functions as a gatekeeper (Guan et al. 2014). Since the skewness and occupancy metrics were first introduced, a multitude of studies have revealed that changes in the global organization of actin can be induced by a range of environmental stimuli, including pathogens (Henty-Ridilla et al. 2013, 2014; Li et al. 2015b; Shimono et al. 2016) and hormone treatments (Pandya-Kumar et al. 2014; Takahashi et al. 2017). Skewness and occupancy metrics have also revealed that some ABP loss-of-function *Arabidopsis* mutants differ in global actin organization compared with wild-type plants, leading to deeper insights into the mode of action of some ABPs in plants (Zhang et al. 2011; van der Honing et al. 2012; Cao et al. 2016).

Skewness and occupancy are thus far the most popular metrics for global analysis of F-actin organization in plants. However, not all aspects of global actin organization can be captured with these two metrics. Software to quantify other parameters that contribute to global F-actin changes in plant cells has been developed. For instance, the software MicroFilament Analyzer, which was first applied to quantify microtubule orientation (Jacques et al. 2013a, 2015), has been used to determine F-actin organization in root cells expressing GFP-fABD2 probes (Jacques et al. 2013b). Moreover, Kimori et al. (2016) recently used shape analysis software based on mathematical morphology to quantify F-actin organization in *Arabidopsis* root hairs. Using their software, shape features such as thickness, multi-orientation index, complexity, and binarized pattern features were extracted from microscopy images of F-actin in roots hairs of root hair defective 3 (*rhd3*) mutants and the wild type (Kimori et al. 2016). Skewness and occupancy rely on skeletonizing and thresholding procedures (Higaki et al. 2010). In certain plant cell types with dense networks of actin filaments, these procedures could prove problematic because some filaments may miss capture. To circumvent this problem, a method was developed that uses fast Fourier transform (FFT) to measure a metric called eccentricity, which is the degree of orientation of F-actin. Using this metric for images of *Physomitrella* expressing Lifeact-monomeric enhanced GFP (mEGFP), it was shown that RHO-of-Plants (ROP) and myosin XI RNA interference (RNAi) knockdown lines had more disordered actin filaments than control lines (Vidali et al. 2010; Burkart et al. 2015).

The methods described thus far typically extract quantitative data from microscopy images obtained from a single or only a few time points. To gain a better appreciation of changes in global actin dynamics over time, induced by a specific treatment or mutation, algorithms were developed that analyze F-actin dynamics from video sequences. These algorithms extract intensity differences and correlation coefficients between two successive images in the video. A steeper decay in the

intensity difference and correlation coefficient is indicative of a more dynamic actin cytoskeleton (Vidali et al. 2010). Using these algorithms, it was shown that global actin dynamics in *Physcomitrella* decreased in response to actin-stabilizing drugs and increased in ROP RNAi lines (Vidali et al. 2010; Burkart et al. 2015). The same algorithm has also been used to demonstrate differences in global actin dynamics between fABD2- and Talin-based probes (Dyachok et al. 2014). Moreover, correlation coefficient analysis on VAEM-derived video sequences showed that global dynamics of the actin cytoskeleton is enhanced in plants responding to microbe-associated molecular patterns (MAMPs) (Li et al. 2015b).

As discussed in the next section, manual measurement of the behavior of individual actin filaments has led to new insights into the mode of action of some ABPs on cortical actin dynamics. However, such manual measurements can be labor intensive (Staiger et al. 2009). To circumvent this problem, the software QuACK (quantitative analysis of cytoskeletal kymograms) was developed to allow semi-automated analysis of cytoskeletal dynamics from video recordings obtained from spinning-disc confocal microscopy or VAEM. This software was based on the analysis of kymograms, which are two-dimensional projections used to represent processes occurring dynamically in a single image (Cvrckova and Oulehlova 2017). Compared with manual tracking of single filaments and correlation coefficients, QuACK has the advantages of speeding up the analysis and enabling estimation of parameters such as dynamic filament end turnover (i.e., maximum event duration parameter) and lateral mobility (maximum lateral displacement) (Cvrckova and Oulehlova 2017).

Recently, an automated image analysis software has been developed that represents F-actin in a network-based framework. This software allows segmentation and quantification of actin structures from two- and three-dimensional image data sets. Using this software, information on the structure of the actin networks and tracking data of Golgi dynamics were combined to uncover a mechanism by which Golgi transport efficiency could be predicted by the global actin topology (Breuer et al. 2017). Use of these recently developed computational tools to study actin dynamics and organization should further advance the field.

### *6.2.3 New Insights on the Mode of Action of Actin-Binding Proteins Gained from Live Cell Imaging*

As indicated above, the tremendous progress toward understanding actin function in plants has been driven by the development of genetically encoded live cell reporters. An area of plant actin research that has probably benefitted most from these live cell tools is deciphering the mode of action of plant ABPs by combining live cell microscopy with genetic and biochemical studies in *Arabidopsis*. Most notable is work from the Staiger group. As noted earlier, Staiger et al. (2009) implemented the use of VAEM to visualize and quantify the behavior of individual

cortical actin filaments in etiolated *Arabidopsis* hypocotyls. Using VAEM as an imaging modality, the ephemeral nature and constant remodeling of cortical actin filaments was elegantly demonstrated and referred to as "stochastic dynamics" (Staiger et al. 2009; Fig. 6.1b, c). This section briefly highlights some of the most compelling findings from work on plant ABPs, focusing specifically on their role in the stochastic dynamic turnover of actin.

*Arabidopsis* ADF4 was the first plant ABP to be analyzed for its impact on actin stochastic dynamics. The *adf4* mutant was shown to have increased hypocotyl lengths compared with wild-type plants when grown in the dark. The hyperelongated hypocotyls of dark-grown *adf4* mutants were characterized by increased F-actin bundling and reduced density. To understand better how loss of *ADF4* function triggered changes in global actin architecture in hypocotyl epidermal cells, single actin filament turnover was examined in wild-type plants and *adf4* mutants. It was found that *adf4* mutants had a threefold decline in severing activity, and an enhancement of filament lengths and lifetimes compared with the wild type (Henty et al. 2011). The decline in severing activity in *adf4* was consistent with the strong actin severing activity of ADF4 in vitro (Tholl et al. 2011; Nan et al. 2017).

The impact of other ABPs on actin stochastic dynamics was also investigated in plants with mutated heterodimeric capping protein (CP) (Li et al. 2012). Work on *cp* mutants and CP overexpressors in *Arabidopsis* uncovered a role for CP in actin severing, filament–filament annealing, and filament elongation that were reminiscent of the effects of AFD4 (Li et al. 2012). Like *adf4* mutants, dark-grown *cp* mutants had longer hypocotyls, whereas CP overexpressors had shorter hypocotyls than the wild type (Li et al. 2014). Furthermore, the end-capping activity of CP was inhibited by phosphatidic acid (PA). It was proposed that CP senses endogenous PA levels and transduces this signal into changes in global actin organization, specifically by modifying the annealing frequency of individual actin filaments (Li et al. 2012). The changes in actin stochastic dynamics induced by MAMPs were mitigated in *cp* mutants, providing genetic evidence that CP participates in innate immune signaling in plants that involves a PA-dependent mechanism (Li et al. 2015b).

Similar approaches to those already discussed were applied to study *profilin1* (*prf1*) mutants. PRF binds G-actin and as a result suppresses nucleation of F-actin (Blanchoin et al. 2014). Like CP and ADF4 proteins, PRF1 was found to specify cortical actin stochastic dynamics in plants but not in the manner expected. Given that PRFs suppress nucleation of actin in vitro, it was surprising to find that nucleation events in *prf1* mutants were reduced in comparison with the wild type (Cao et al. 2016). A likely explanation for the reduced nucleation frequency in *prf1* is that PRF1 modulates the activity of the actin nucleator formin (Michelot et al. 2005; Cao et al. 2016).

It is clear from the above studies that live cell imaging combined with genetics provides a powerful tool for dissecting the function of ABPs in remodeling the actin cytoskeleton. However, a recent study demonstrated the elegance of biochemical and phylogenetic approaches for gaining deeper insights into mechanisms by which ABPs modulate actin dynamics and the divergence in biochemical function for

some of these proteins. Nan et al. (2017) showed that the 11-membered ADF family in *Arabidopsis* can be grouped into two categories based on their biochemical effects on actin. The D-type, which consist of nine ADFs, has depolymerizing activity whereas the other two ADFs, namely ADF5 and ADF9, have bundling activity and belong to the B-type. It was shown that the N-terminal extensions, together with several conserved mutations, led to the divergent biochemical activities of plant ADFs (Nan et al. 2017). The bundling activity of ADF5 appears to play a major role in pollen germination and pollen tube expansion (Zhu et al. 2017).

## 6.3 Novel Plant Proteins Linked to Actin and Their Biological Functions

In a recent article, Gunning et al. (2015) presented a hypothesis on the specialization of actin and ABPs during evolution. They proposed that the expansion in the number of genes encoding for actin and actin-related proteins in plants compared to other eukaryotes could explain the distinct and functionally specialized actin networks in plants. For example, *A. thaliana* and soybean (*Glycine max*) have 12 and 17 cytoplasmic actin genes, respectively, compared with only two in the human or *Drosophila melanogaster* genomes. The number of ADFs range from 12 in *Arabidopsis* to 27 in *Musa acuminata*; in fungi and other metazoans, this number ranges from one to three. Based on these observations, it was proposed that such diversity in actin isoforms could confer specificity in their interaction with ABPs, while also suggesting the coevolution of actin and ABPs in plants (Gunning et al. 2015). Indeed, such a scenario was demonstrated in a study published a little more than 10 years ago by the Meagher group. Plant actin genes have been grouped into two classes, namely vegetative and reproductive, based on their expression patterns (Meagher et al. 1999). Misexpression of *ACTIN1* (*ACT1*), a reproductive actin using the vegetative *ACTIN2* (*ACT2*) promoter, resulted in dwarfed plants and severely disrupted actin organization. Coexpression of reproductive ADF and PRF isovariants with the misexpressed ACT1 rescued the dwarf phenotype, suggesting that plant actin and some ABPs coevolved to confer specialized cellular functions (Kandasamy et al. 2007). The possibility that ABPs instead of actin confer functional specialization in plants was shown by the ability of cytoplasmic actins from protists and humans to rescue the growth phenotypes of *Arabidopsis* vegetative actin mutants (Kandasamy et al. 2012).

In this section, we review recently discovered plant proteins that have been linked directly or indirectly to actin; these proteins may have also coevolved with plant actin to confer functionally specialized cellular actin networks in plants. We begin by briefly introducing novel plant ABPs that play a role in organelle movement, stomatal gating, nodulation, and tip growth. Without going in depth into plant myosins, we present exciting results on new myosin-binding proteins that have been identified in plants and probably function as myosin receptors and adaptors.

The discovery of these myosin-binding proteins represents a breakthrough, not only for the plant cytoskeleton but also for the eukaryotic cytoskeleton as a whole, because myosin receptors and adaptors have so far remained elusive in other popular eukaryotic models such as yeast (Peremyslov et al. 2013, 2015; Stephan et al. 2014; Citovsky and Liu 2017; Kurth et al. 2017). In Sect. 6.3.3, we also touch on newly identified plant-specific proteins that link actin to endomembranes.

### *6.3.1 Novel Plant Actin-Binding Proteins*

Plant ABPs with homologs in other eukaryotes have conserved actin-binding domains (ABDs) that are crucial for directly interacting with actin. For example, fimbrins have ABD1 and ABD2, which contain tandemly arranged calponin-homology domains, whereas formins contain the formin homology (FH) domain (Sheahan et al. 2004; Wang et al. 2004; Michelot et al. 2005; Dong et al. 2013; Peremyslov et al. 2013). With completion of the *Arabidopsis* genome, early progress toward understanding the biological function of many of the conserved plant ABPs summarized in Table 6.1 was made using reverse genetics and biochemical characterization of the purified ABPs (Christensen et al. 1996; Kandasamy et al. 2002; Cheung and Wu 2004; Michelot et al. 2005; Huang et al. 2005). By contrast, some of the more recently identified plant ABPs were discovered through forward genetic screens. THRUMIN1 is one example; it is an actin bundling protein discovered through a forward genetic screen for *Arabidopsis* mutants with defects in chloroplast movement. Although the domains that are crucial for THRUMIN1–actin binding have yet to be determined, recombinant THRUMIN1 can bundle actin, and a THRUMIN1-YFP fusion was shown to decorate filamentous networks in plants that were sensitive to the actin depolymerizing drug, latrunculin B (LatB) (Whippo et al. 2011).

Blue light-triggered chloroplast movement is orchestrated by a population of actin filaments called cp-actin (chloroplast-actin), which are found at the chloroplast periphery and plasma membrane (Kadota et al. 2009). Like THRUMIN1, the proteins CHLOROPLAST UNUSUAL POSITIONING 1 (CHUP1) and KINESIN-LIKE PROTEIN FOR ACTIN-BASED CHLOROPLAST MOVEMENT (KAC) were discovered in *Arabidopsis* through forward genetic screens for defects in light-induced chloroplast translocation. CHUP1 and KACs are conserved in plants and involved in regulating cp-actin-mediated chloroplast movement (Oikawa et al. 2003; Suetsugu et al. 2010, 2012). CHUP1 contains an actinin-type ABD (Oikawa et al. 2003), whereas the C-terminus of KAC interacts with F-actin in vitro (Suetsugu et al. 2010). Recently, a model for the blue-light avoidance response in chloroplasts was proposed in which CHUP1, KAC, and THRUMIN1 function in cp-actin-dependent and independent pathways (Suetsugu et al. 2016).

STOMATAL CLOSURE-RELATED ACTIN BINDING PROTEIN 1 (SCAB1) was also discovered from a forward genetic screen. In the case of SCAB1, mutants with faster rates of water loss in detached leaf assays were isolated and it was found

that the inability of *scab1* to prevent excessive water loss was caused by impaired stomatal function. Using high-speed cosedimentation assays and imaging of truncated SCAB1-GFP fusions, SCAB1 was shown to bind actin through the N-terminal residues 54–148, which correspond to the central alpha-helical regions of the protein (Zhao et al. 2011). Follow-up studies on the crystal structure of SCAB1 revealed that the protein forms a dimer via its coiled-coil domains and is a bivalent F-actin crosslinker. Furthermore, a pleckstrin-homology (PH) domain at the SCAB1 C-terminus implicates phosphoinositides in SCAB1-mediated actin-mediated stomatal function (Zhang et al. 2012). Interestingly, another forward genetic screen for *Arabidopsis* mutants that lose water faster has led to the recent discovery of CASEIN KINASE 1-LIKE PROTEIN 2 (CKL2) as a component of actin-mediated stomatal movements. CLK2 physically interacts with ADF4 and phosphorylates it. Although biochemical studies of recombinant CLK2 show that it does not bind or bundle actin in vitro, a CKL2-GFP fusion decorated F-actin in guard cells and other plant cell types (Zhao et al. 2016). The identification of SCAB1 and CLK2 reinforce previous studies demonstrating the crucial role of the actin cytoskeleton in the modulation of stomatal function (Higaki et al. 2010; Li et al. 2013).

Forward genetic screens identified another novel protein with potential actin-interacting properties, SCAR-Nodulation (SCARN). SCARN was discovered by characterizing nodulation-defective mutants in the model legume *Lotus japonicus*. Although SCARN has domains with similarity to the SUPPRESSOR OF cAMP RECEPTOR (SCAR) proteins, which are components of the ARP2/3 actin nucleating complex, it is larger than the four *Arabidopsis* SCAR proteins and is only 26% and 30% identical to *A. thaliana* SCAR2 and SCAR4, respectively (Qiu et al. 2015). Biochemical characterization and *in planta* localization of SCARN have yet to be conducted. However, the presence of the SCAR homology and Wiskott-Aldrich homology 2 (WH2) domains strongly indicates that it interacts with actin (Fig. 6.3) (Qiu et al. 2015). Interestingly, THRUMIN1 and SCAB1 also contain the actin-binding WH2 domains found in many conserved ABPs and in other putative plant actin–endomembrane linker proteins (Fig. 6.3). The discovery of SCARN and its potential role in nodulation is consistent with previous mutant and cell biological studies implicating proteins belonging to the ARP2/3 complex as components of the cellular machinery for infection thread formation and nodule development (Miyahara et al. 2010; Hossain et al. 2012; Gavrin et al. 2015).

CROLIN1, another plant protein that binds actin in vitro, was identified not through the forward genetics route but via a bioinformatics search for proteins with predicted actin crosslinking domains. CROLIN1 has a similar structure to FASCIN, an animal ABP (Jawhari et al. 2003). However, the actin crosslinking domains of CROLIN1 are only 16% identical to those of FASCIN (Jia et al. 2013). There are six CROLIN genes in the *Arabidopsis* genome; CROLIN1 is expressed specifically in pollen, suggesting that it might be part of the cytoskeletal machinery for maintaining tip growth (Jia et al. 2013). Although the importance of CROLIN1 in tip-growing plant cells has yet to be functionally characterized, the role of actin and

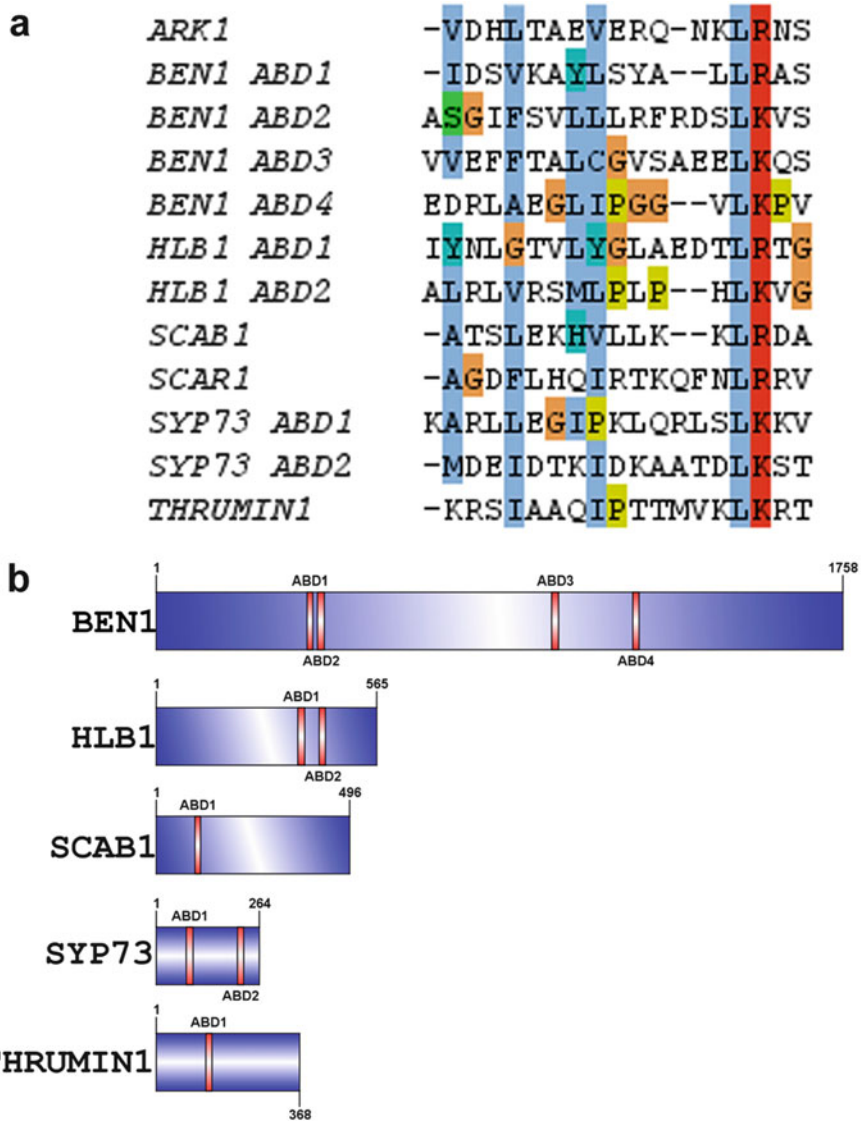


**Fig. 6.3** Plant ABPs with WH2 domain. (**a**) Amino acid sequence alignment of WH2 domains in various plant actin-binding proteins. The WH2 domain consists of 17–19 amino acids and contains several highly conserved residues at specific positions in the motif. The amino acid sequences were aligned using the MUSCLE algorithm and the residues are color coded with ClustalX according to their similarity at that position. (**b**) Scheme showing the domain organization of selected ABPs with WH2 domains (labeled as ABD1-4). The Eukaryotic Linear Motif program was used to predict the location of WH2 domains in various plant actin-binding proteins. There appears to be no pattern with regard to the number or location of the WH2 actin-binding motif within the proteins

ABPs in this process is well established (Su et al. 2012; Wu et al. 2012; Rounds and Bezanilla 2013; Stephan et al. 2014; Huang et al. 2015; Wang and Hussey 2015).

A recently described protein involved in tip growth that directly interacts with actin is MICROTUBULE-ASSOCIATED PROTEIN 18 (MAP18). From its name, MAP18 was originally described as a microtubule binding protein that destabilizes microtubules during pollen tube elongation (Wang et al. 2007). However, MAP18 has F-actin severing activity that is essential for maintaining pollen tube growth direction (Zhu et al. 2013). The severing activity of MAP18 is also required to keep the nucleus at a relatively fixed distance from the apex of a growing root hair (Zhang et al. 2015), which might play an important role in root hair development (Ketelaar et al. 2002; Jones and Smirnoff 2006). MAP18 is one of several proteins that directly bind to actin microfilaments and microtubules, facilitating interactions between these two major cytoskeletal components during plant development. Other proteins with such activity include formins, MICROTUBULE-DESTABILIZING PROTEIN 25 (MDP25), SB401, ARPC2, kinesin-like calmodulin binding protein (KCPB), and myosin VIII (Huang et al. 2007; Deeks et al. 2010; Qin et al. 2014; Wu and Bezanilla 2014; Havelkova et al. 2015; Tian et al. 2015; Sun et al. 2017). Interestingly, MDP25 has F-actin severing activity that is reminiscent of the severing activity of other pollen tube tip-localized proteins such as ROP-INTERACTIVE CRIB MOTIFF-CONTAINING PROTEIN 1 (RIC1). The biochemical activities of RIC1 and MDP25 strongly support a crucial role for actin severing in remodeling the actin cytoskeleton during maintenance of tip growth (Qin et al. 2014; Zhou et al. 2015). The interaction between microtubules and F-actin during plant development, and detailed characterization of proteins that mediate these interactions, are fruitful areas for future research (Petrasek and Schwarzerova 2009).

### *6.3.2 Myosin Receptors and Adaptors in Plants*

The continuous and rapid movement of organelles and vesicles along F-actin tracks in plants is historically referred to as cytoplasmic streaming and relies on highly conserved myosin motors (Avisar et al. 2008, 2012; Madison and Nebenfuhr 2013; Ueda et al. 2015; Citovsky and Liu 2017). Genetics studies have shown the importance of myosins in a range of plant physiological processes, including organ straightening, gravitropism, pollen tube and root hair growth, flowering time, and maintenance of normal plant stature (Ojangu et al. 2007; Peremyslov et al. 2008, 2010, 2015; Park and Nebenfuhr 2013; Madison et al. 2015; Okamoto et al. 2015; Talts et al. 2016). However, the molecular mechanisms by which myosin-driven intracellular plant transport networks regulate these processes remain unclear.

Perhaps the most exciting finding regarding myosins, which is beginning to shed light on the phenomenon of cytoplasmic streaming, was the discovery of plant proteins that function as myosin receptors and adaptors. In a two-hybrid screen

using the *Arabidopsis* myosin XI-k globular tail domain (GTD) as bait, Peremyslov et al. (2013), identified a family of previously uncharacterized proteins with a highly conserved coiled-coil domain of unknown function (DUF593) and a transmembrane domain. This protein family (MyoB) has 16 members in *Arabidopsis* and some have been shown to bind to myosin through their conserved DUF593 domain (Kurth et al. 2017; Peremyslov et al. 2013). Live cell imaging of MyoB-GFP fusions revealed association of the fusion with motile vesicles that colocalized with myosin XI-k. Surprisingly, MyoB-GFP-decorated vesicles had only minimal overlap with known post-Golgi markers and organelles (Peremyslov et al. 2013). Detailed analysis of MyoB dynamics showed faster rates of MyoB vesicle movement compared with other organelles, which led to models proposing that larger organelles and other secretory vesicles are passively carried by MyoB-myosin-driven cytoplasmic streaming (Peremyslov et al. 2013, 2015). A member of the MyoB receptor family called RAC5 INTERACTING SUBAPICAL POLLEN TUBE PROTEIN (RISAP) was identified independently in tobacco pollen tubes, localized to trans-Golgi network (TGN) compartments at the apical dome (Stephan et al. 2014).

The model proposed by Peremyslov et al. (2013, 2015) was supported by the recent discovery of a two unrelated protein families that also bind to myosin. Unlike the majority of MyoBs, these proteins did not contain a transmembrane domain. As such, they were named myosin adaptor of family A (MadA) and family B (MadB). Furthermore, although MyoB was associated exclusively with motile vesicles, MadA and MadB proteins partitioned between the cytoplasm and vesicles. Interestingly, MadA1 localized to the nucleus (Kurth et al. 2017). A forward genetic screen for mutants with abnormal nuclear shapes discovered a nucleocytoplasmic linker consisting of myosin XI-i and an outer-membrane-localized WPP domain-interacting tail-anchored protein (WIT) (Tamura et al. 2013). MadA1 and the myosinXI-i–WIT complex could be components of the molecular machinery that enables nuclear positioning and/or nucleocytoplasmic transport in plants (Tamura et al. 2013; Kurth et al. 2017). These seminal findings pave the way for exciting studies on actomyosin-driven transport pathways in plants.

### *6.3.3 Plant Proteins that Facilitate Actin–Endomembrane Crosstalk*

The endomembrane system comprises a network of interconnected organelles with related and coordinated functions. This system is crucial for plant development and various intra- and intercellular signaling processes (Surpin and Raikhel 2004). Plants rely on this dynamic network of internal membranes for proper processing, modification, and transport of their cytosolic components. Important constituents of the endomembrane system are the plasma membrane, endoplasmic reticulum (ER),

Golgi apparatus, TGN, vacuole, and nuclear envelope, as well as chloroplasts, peroxisomes, and mitochondria. These membrane system components interact with each other to coordinate cellular trafficking and cell morphogenesis during different stages of plant development (Surpin and Raikhel 2004; Vukašinović and Žárský 2016). Several components responsible for endomembrane system organization and its functionality have been discovered (Kim and Brandizzi 2016; Angelos et al. 2017; Brandizzi 2017; Wang et al. 2017c). In recent years, however, much attention has focused on cytoskeleton–endomembrane crosstalk, as there is accumulating evidence that the interaction between these two cellular systems has biological significance for many aspects of plant cell function and development (Hussey et al. 2006; Sampathkumar et al. 2013).

Some components that mediate actin–endomembrane interaction in plants (including CHUP1, KAC, and CP) have been discussed in previous sections (see Table 6.1 and Sects. 6.2.3 and 6.3.1). But, perhaps the most important breakthrough in the past 5 years was the discovery of a large family of plant-specific actin-membrane adaptors called the NETWORKED (NET) family of proteins. NET proteins use their conserved N-terminal domain to bind actin and, through their variable C-terminal sequences, they form links with different membrane compartments in the cell. Some members of the NET family have been characterized biochemically, and mutant studies indicate that they function in actin-related biological processes such as cellular expansion in roots and pollen development (Deeks et al. 2012; Wang et al. 2014; Wang and Hussey 2017). Their role as linkers between actin and distinct membrane components has been covered in recent reviews; therefore, the NET proteins are not discussed extensively here. Instead, we refer the reader to some recent reviews (Hawkins et al. 2014; Wang and Hussey 2015; Wang et al. 2017a, b). Future studies using combinatorial mutants and live cell microscopy should shed light on the biological function of this interesting family of plant-specific ABPs.

Although NET proteins have recently received the most attention as actin–endomembrane linkers, other candidate proteins that probably function in a similar manner are also being discovered. For example, in a recent forward genetic screen, Sparks et al. (2016) looked for *Arabidopsis* mutants that showed differential sensitivity to LatB. Given that LatB is an actin-disrupting drug, it was expected that such screens would discover new proteins that might be important for plant actin function. One recessive mutant called *hypersensitive to LatB1* (*hlb1*) because of its heightened sensitivity to LatB was disrupted in a plant-specific gene encoding a protein with tetratricopeptide repeats (TPR) and a conserved C-terminal domain with similarity to phosphoinositide-binding PH domains. Interestingly, HLB1 localized to the TGN and was found to interact with an ADP-ribosylation factor (ARF)—a guanine nucleotide exchange factor called HOPM INTERACTOR 7/BREFELDIN A-VISUALIZED ENDOCYTIC TRAFFICKING DEFECTIVE 1 (MIN7/BEN1) (Tanaka et al. 2009, 2013; Nomura et al. 2011). Subsequent cell biology and genetic studies indicated that HLB1 together with MIN7/BEN1 could

be an important component of the molecular machinery by which actin coordinates exo- and endocytic trafficking pathways in plants (Sparks et al. 2016).

Another noteworthy study was made by Cao et al. (2016), who used a bioinformatic approach to identify plant proteins that had similar domains and functional attributes as the CLIMp-63/p63 proteins, which are anchors between the ER and the cytoskeleton in mammals. They also paid close attention to plant proteins that contain both the CLIMp-63/p63 membrane anchor and cytoskeletal binding domains. In doing so, a family of unique soluble NSF attachment protein receptor (SNARE) proteins with three members (SYP71, SYP72, and SYP73) was identified. Consistent with predictions from bioinformatics analysis, SYP73-GFP localized to the ER in transient expression assays. Interestingly, in cells overexpressing SYP73-GFP, the fusion assumed a pattern that resembled actin cables; direct binding to actin was proven in high-speed cosedimentation assays. The study indicated that SYP73 could represent a novel actin–ER linker that has a different role from conventional plant SNARE proteins (Cao et al. 2016).

HLB1, MIN7/BEN1, and SYP23 contain the WH2 domains predicted to bind actin (Fig. 6.3). However, unlike SYP73, direct binding of HLB1 and MIN7/BEN1 to actin have yet to be demonstrated by biochemical approaches, although it was shown that HLB1 TGN compartments track along F-actin (Sparks et al. 2016). The *hlb1*, *min7*/*ben1*, and *syp73* mutants have defects in early plant development and are hypersensitive to LatB (Cao et al. 2016; Sparks et al. 2016). Whereas loss of *HLB1* and *MIN1*/*BEN7* function inhibit plant development by impairing secretion and endocytosis, respectively (Sparks et al. 2016; Tanaka et al. 2009, 2013), SYP73 exerts its effects on plant growth through mechanisms that involve actomyosin-mediated ER remodeling (Cao et al. 2016).

As noted earlier, one pivotal role for actin in plants is the regulation of tip growth. Genetic evidence for this comes from the observation that mutation of the root hair-expressed vegetative actin isoform ACT2 (*act2*) causes distorted root hairs (Gilliland et al. 2002; Nishimura et al. 2003; Ringli et al. 2002). In this regard, forward genetic screens for *Arabidopsis* mutants that resemble *act2* led to the discovery of an ARF-GTPase activating protein (GAP) called *agd1* (for *arf-gap-containing domain*) (Yoo et al. 2008). The *agd1* mutants function in overlapping pathways with *act2* that involve phosphoinositide metabolism (Yoo et al. 2012). Detailed studies of an AGD1-GFP fusion revealed that AGD1 localizes to distinct domains of the plasma membrane through its phosphoinositide-binding PH domains, leading to the hypothesis that this plant ARF-GAP protein might be involved in maintenance of normal actin dynamics in plants, similar to mammalian models (Yoo et al. 2017). Indeed, root hairs of *agd1* mutants have altered actin dynamics (Yoo et al. 2008). Future studies on HLB1, MIN7/BEN1, AGD1, and SYP73 in mediating actin–endomembrane crosstalk present exciting opportunities for future research.

## 6.4 Emerging Roles of the Actin Cytoskeleton in Plants

In our discussion of live cell imaging tools and ABPs in Sects. 6.2 and 6.3, we touched on a number of plant biological processes in which the actin cytoskeleton plays a crucial role. For example, we presented evidence for the involvement of plant ABPs in plant responses to pathogens, a topic that has been discussed in some recent review articles (Day et al. 2011; Porter and Day 2016; Inada 2017). Moreover, we briefly discussed actin involvement in nodulation when we introduced the SCARN protein (Qiu et al. 2015). In this section, we introduce other biological processes in which there is recent compelling evidence for actin involvement.

In keeping with the theme of plant–microbe interactions, we begin this section by highlighting a study by Yang et al. (2017) showing that actin might facilitate the movement of *Agrobacterium tumefaciens* virulence (VIR) factors for delivery of transfer (T)-DNA into recipient cells. One protein component of the *Agrobacterium* VIR type IV secretion system (Fronzes et al. 2009) is VIRE2, which has a nuclear localization signal that directs its import to the nucleus (Citovsky et al. 1992, 1994). Using a method based on split-GFP, Yang et al. (2017) showed that VIRE2 associates with the ER and actomyosin system to traffic into the plant host cells. Given that *Agrobacterium* is a major tool for plant genetic modification, new knowledge about how it hijacks the host cytoskeletal and endomembrane system to enable T-DNA integration could lead to new strategies for more efficient plant transformation techniques (Yang et al. 2017).

From our discussion of the SCAB1 and CLK1 proteins, it is clear that actin plays a crucial role in stomatal movement. A study of microcompartmentation in *Arabidopsis*, a process in which soluble proteins are distributed within subcellular compartments in a nonhomogeneous manner, discovered a potential role for the interaction between the enzyme fructose bisphosphate aldolase (FBA8) and F-actin in stomatal function. FBA8 has two ABDs and cosediments with polymerized F-actin. Although fluorescence lifetime imaging microscopy (FLIM) suggested that FBA8-F-actin interaction in vitro is biologically relevant, colocalization results were inconclusive (Garagounis et al. 2017). Nonetheless, guard cells of *fba8* mutants had minor alterations in F-actin organization and slightly reduced rates of stomatal closure in response to low humidity (Garagounis et al. 2017). It remains to be determined how FBA-8 fits into actin-mediated stomatal gating mechanisms that involve SCAB1, CLK2, and the ARP2/3 complex.

Recent studies in *Arabidopsis* are also beginning to shed light on hormonal regulation of the actin cytoskeleton. It has been shown that indole-3-acetic acid (IAA), endogenous auxin, and some of its analogs can trigger reorganization of the actin cytoskeleton (Rahman et al. 2007; Dhonukshe et al. 2008; Nick et al. 2009; Nick 2010; Zhu and Geisler 2015). Recent studies have provided new mechanisms by which auxin facilitates actin-mediated modification of plant cell expansion. One mechanism involves a process that was covered earlier, namely actin–endomembrane crosstalk. Some of the NET proteins alluded to earlier link actin to the vacuole (Deeks et al. 2012). By imaging a Lifeact F-actin and vacuolar

reporter, Scheuring et al. (2016) showed that auxin-induced expansion and constriction of the vacuole is dependent on actin. This auxin-mediated control of the volume of the vacuole was proposed to have an indirect impact on the size of the cytoplasm, which in turn influences the ability of the cell to expand (Scheuring et al. 2016). In another study, Takahashi et al. (2017) provided genetic and cell biology evidence that the auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D) remodels the actin cytoskeleton, in part through small acidic protein 1 (SMAP1) (Takahashi et al. 2017). It remains to be determined how SMAP1 is linked to actin-dependent remodeling of the vacuole to coordinate plant cell expansion.

In addition to auxin, inhibitors of polar auxin transport such as tri-iodobenzoic acid (TIBA) and naphthylphthalamic acid (NPA) can disorganize the actin cytoskeleton (Dhonukshe et al. 2008; Rahman et al. 2007; Zhu and Geisler 2015). In studies that mapped the NPA-binding site of the ABCB chaperone protein TWISTED DWARF 1 (TWD1), a new mechanism by which auxin transport inhibitors modify actin dynamics was proposed. Although TWD1 interacted with ACT7, albeit indirectly, genetic and cell biology evidence support a scenario in which NPA triggers actin reorganization by binding to TWD1 (Zhu et al. 2016).

Most of the examples discussed in this chapter have focused on *Arabidopsis*. However, mutant work using other plant species is now pointing to traits controlled by actin genes that could have adaptive value. One notable example is work on the bumble bee-pollinated monkeyflower (*Mimulus lewisii*). A dominant-negative mutation in an actin gene resulted in substantial reduction in flower corolla tube length. The actin mutant plants with altered corolla length had a 70% reduction in bumble bee visitation rates compared with wide-type plants, suggesting that actin genes might shape the evolution of ecologically important traits (Ding et al. 2017). Although additional work is needed to determine whether mutations in actin and ABPs can explain the variations in plant architecture that occur in nature, a recent association study in wild pearl millet (*Pennisetum glaucum*) revealed that phenotypic variation in flower number under different rainfall conditions was the result of single nucleotide polymorphisms on the *myosin XI* gene (Ousseini et al. 2017).

## 6.5 Conclusions and Perspectives

The actin cytoskeleton controls essential growth and developmental processes in plants, from cell division and expansion to responses to biotic and abiotic stresses. In the past decade, a flurry of research on actin function in plants has led to new knowledge on the mode of action of some plant ABPs, novel actin–endomembrane protein linkers, and discovery of the elusive myosin receptors and adaptors. The latter advance in particular has drastically altered our thinking about an age-old process, namely, how plants rapidly move their cellular components through cytoplasmic streaming. Although much progress in understanding actin in plants was brought about by the development of genetically encoded fluorescent reporters and microscopy modalities to analyze actin dynamics, standard forward genetics in model plants and intensive yeast two-hybrid screens continue to deliver exciting results.

Historically, plant cell biologists have refined methods used for research in other eukaryotic models to address their questions of interest. This trend is expected to continue for future research on plant actin cytoskeleton. For example, pharmacological approaches using actin-disrupting compounds such as latrunculin and cytochalasin are an essential component of the toolkit for dissecting actin functionality in plants (Baluška et al. 2001; Wang and Nick 1998). New compounds with latrunculin-like effects are being isolated (Filipuzzi et al. 2017) that could be used in tandem with cellular, genetic, and biochemical methods. The use of compounds with actin-modifying properties can yield surprising and novel results with regard to understanding basic plant physiological processes (Toth et al. 2012). Although cell-permeable compounds are valuable tools for research on actin function, they are limited in that they do not allow for cell type-specific actin disruption. We have seen under certain situations that some genetically encoded live cell fluorescent protein reporters can lead to plant growth and developmental effects reminiscent of actin-perturbing compounds. Recently, such observations have been exploited to develop new tools for perturbing actin function within single cells (Harterink et al. 2017); it would be exciting to see how such technologies can be applied to plant actin research.

The role of the cytoskeleton in many fundamental cellular processes dictates that deleterious mutations in genes that regulate its function are subject to negative selection. Although this is mostly true for mammals and yeast, mutations in plant cytoskeletal genes often result in only minor developmental defects (Gunning et al. 2015). Because plants are sessile, they have probably evolved a larger repertoire of actin and actin regulatory genes to enable them to adapt to the environment. Some genes encoding proteins that regulate cytoskeletal function have now explained natural variation in plants (Ousseini et al. 2017; Rishmawi et al. 2014). Many of the compounds that perturb actin dynamics are natural products synthesized by organisms, and it is likely that more remain to be discovered. Although highly speculative, one of the drivers that might have shaped the evolution of plant actin genes is the diversity in chemical compounds secreted by microbes and plants themselves. With advances in genomics technologies, this is certainly an area ripe for testing.

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# Chapter 7
# Cell Wall Expansion: Case Study of a Biomechanical Process


**Alexis Peaucelle**

**Abstract** The secret of plant biomechanical growth control lies in the ability of plants to expand the cell wall without bursting. This chapter discusses various views on plant cell growth. We try to show the multiples processes leading to growth and the redundant functions that different components of the cell wall display during the growth process.

## 7.1 Basics of Plants Tissue Mechanics

### *7.1.1 Generalities*

From the dawn of humanity, the diversity of mechanical properties exhibited by plant tissues were explored in tool-making, fabrics, houses, furniture, and cutlery. Even the discovery of artificial polymers did not entirely replace plant-derived materials such as linen and cotton. This chapter focuses only on plant tissue mechanics and its link to growth: the interplay between organogenesis and the mechanics of the primary cell wall. The main characteristic of plant cells is the presence of a cell wall, which is a rigid pectocellulose hydrogel encapsulating every single plant cell. The cell wall forms a protective layer and provides structural support for the cell, generating unique properties of the plant tissue as well as strong constraints on cell growth.

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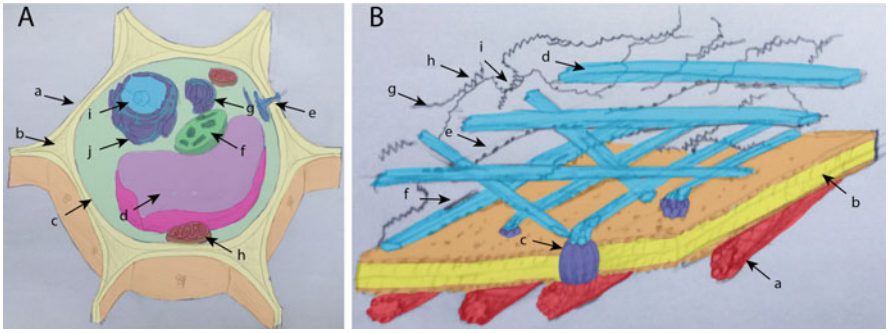


**Fig. 7.1** Cell wall structure: (**a**) Organization of a plant cell, showing (a) cell wall, (b) middle lamella, (c) plasma membrane, (d) vacuole, (e) plasmodesmata, (f) chloroplast, (g) Golgi apparatus, (h) mitochondria, (i) nucleus, and (j) endoplasmic reticulum. (**b**) Structure of the primary cell wall, showing (a) microtubules, (b) plasma membrane, (c) cellulose synthase complex, (d) cellulose microfibril, (e) hemicellulose, (f) xyloglucans, (g) pectin, (h) demethylated homogalacturonan, and (i) methylated homogalacturonan

### *7.1.2 Basis of Plant Cell Wall Mechanics*

The first thing you see in a plant tissue is the cell wall, as Robert Hook's historical description in his book *Micrographia* so remarkably demonstrated. This hydrogel, delimited by a membrane, surrounds the protoplast with its nucleus, mitochondria, chloroplasts, and vacuole (Fig. 7.1). The protoplast exerts a pressure on the cell wall. A good metaphor is a bicycle tire and its tube. If you remove the pressure, cells collapse and the plant loses its shape. In some tissues, a process known as secondary cell wall thickening dramatically increases cell wall rigidity. In such tissues, turgor pressure is not required to maintain organ shape. For more information, read Busse-Wicher et al. (2016). Here, we focus on the primary cell wall, which is able to undertake expansion and growth.

Another metaphor that helps in understanding the growth process in plants is the growing classroom: to expand a classroom in a brick building you need to extend the walls. For that, you must push on the walls and add new bricks or reshuffle the existing bricks into a less compact structure. As with the tire metaphor, it helps in understanding the huge tension exerted on the cell wall and the energy needed to expand the cell wall. If the cell wall loses its integrity or the turgor pressure is too high, the cell bursts and the plant collapses (Fig. 7.2). How the cell wall manages to expand without losing its integrity is an extraordinary biophysical puzzle that is explored in this chapter.

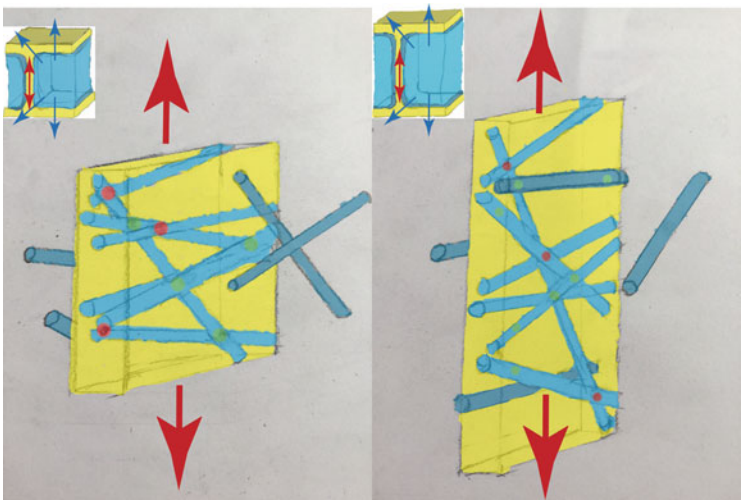

**Fig. 7.2** Changes in the cell wall during growth: (**a**) The cell wall expands under the tension of turgor pressure (blue arrows). Local rearrangement of the cell wall along labile like (green) permits rearrangement of the cell wall. Red arrows indicate the direction of expansion. (**b**) Synthesis in situ and exocytosis of new cell wall components (purple elements) and the change in cell wall links to more rigid ones (red circles) prevent cell wall bursting

## 7.2 What Is Growth?

### *7.2.1 Definition*

First, let us define growth as an irreversible extension of the cell. If we compare plant tissue to an inert material (with no biological activity), the extension can be described as plastic. For a tissue to expand, the cell walls must expand through rearrangement of existing cell wall components or synthesis of new material. To describe the process of growth, we need to measure three parameters simultaneously (Boyer et al. 1985):

- *Turgor pressure*: This is the force that pulls the cell walls apart. This pressure originates from the highly concentrated water in the cytoplasm and vesicles of the cell. The hydrophilic molecules in the cytoplasm attract water that flows freely in and out of the cells and from cell to cell through aquaporins (pores in the plasma membrane) or the plasmodesmata (cell-to-cell cytoplasmic junctions).
- *Cell wall mechanics*: Here we determine how much energy is needed to expand the existing cell wall (elasticity) and how much it can reshuffle itself (plasticity).
- *Synthesis of new material*: Growth involves exocytosis of new cell wall material and cell wall synthetizing enzymes.

To measure all these parameters simultaneously is very difficult and has not been achieved so far (with the exception of pollen tubes). We discuss the technical challenges one by one in the following sections.

### *7.2.2 Is Growth Really a Mechanical Problem?*

For a long time, organogenesis was studied by tracking cells throughout cell division and neglected the cell expansion aspect of organogenesis. New organs correlate with new cell division patterns. For example, new organ formation in the meristem, or later in the root, is always associated with periclinal division in the deep layer of the tissue (Walles 1991). In some cases, the whole process of organogenesis can be described as a series of organized symmetric or asymmetric divisions (Gunning et al. 1978). Studies have demonstrated how important the cell division pattern is for organogenesis. In the early 1990s, a series of experiments measuring the increasing or decreasing cell division rate in elongating tissue showed that the rate had little or no effect on organogenesis (Wyrzykowska et al. 2002). This brought back the old idea that cell mechanics, rather than cell number, controls growth. However, cell division and cell expansion are linked; cell division is under the control of cell expansion. It is possible to predict cell division in the meristem by its increase in size and the mechanical stress it is experiencing (Jones et al. 2017). In other words, cell wall expansion prefigures the division pattern that follows the cell structure achieved by growth. Therefore, we could settle on the idea that growth and organogenesis in plants is driven by cell wall expansion.

Yet, a recent study has shown that the levels of cell wall synthesizing enzyme in the meristem are cell-division controlled (Yang et al. 2016). Thus, there is mutual control: growth-associated changes in cell wall mechanics could be under the control of the cell division process.

**Summary** Cell division and growth are linked through the following feedback loop: cell wall mechanics controls cell expansion, which controls cell division, which in turn affects cell wall mechanics.

## 7.3 Modeling and Mathematical Approaches

The elements that control cell wall expansion are clearly part of a complex process. To grasp a complex problem, it is often helpful to propose a simple mathematical equation with the minimum number of possible variables. This was most clearly stated by Lockhart (1965), who proposed a biophysical equation for the mechanical control of growth of the cell wall:

$$\text{Rate} = m\,(\Psi p - Y)$$

The growth rate is proportional to the turgor pressure ($\Psi p$) and extensibility ($m$) above the yield threshold ($Y$).

Behind this mathematical statement is the following idea. The pressure is associated with two parameters that describe cell wall mechanics: its capacity to expand irreversibly ($m$) and a threshold ($Y$). The existence of a threshold represents the ability of plant tissue to halt growth without stopping synthesis or to reduce the turgor pressure to zero.

Defining these parameters and estimating their numerical values is quite challenging. The extensibility $m$ is a complicated parameter to determine. In Lockhart, $m$ stands for all the parameters that permit expansion: synthesis of new cell wall components and extension of the existing ones. Which of the two parameters is most important is the subject of a debate that is polarizing the scientific community. In creep experiments, which determine cell wall remodeling under tensile stress, $m$ is often reduced to plastic deformation. As discussed above, synthesis of cell wall components should also be considered in irreversible expansion and is a component of the $m$ factor.

Another way of evaluating parameters is through computer simulation. Since the Lockhart publication, a series of models describing organ growth have been proposed. These models always face the geometric problem and a huge number of unknown parameters (e.g., thickness of the cell wall and synthesis rate). To date, some successful models have managed to describe growth in two dimensions (2D).

Anja Geitmann (Parre and Geitmann 2005) was the first to propose a reliable model for pollen tube growth. The pollen tube is a cell presenting very rapid tip growth; its goal is to project the sperm cell situated at the tip of the tube into the ovule and thus grows through the pistil. The most recent models take into account changes in the local geometry of the cell wall over time. They are able to simulate the transient oscillatory growth in different pollen tube species observed in nature. In Geitmann's work, the minimum number of parameters for the model were measured directly. To best fit reality, "guessing" the different parameters of the equation was associated with real measurement of cell wall elasticity, cell wall synthesis (exocytosis rate), turgor pressure, and growth using high temporal resolution.

**Summary** Modeling helps to test and evaluate the importance of different elements in growth. The most informative models are the simplest ones that can describe the observed growth based on the minimum number of variables. The best studies also associate the evaluation of parameters with in situ measurement.

## 7.4 Measuring Turgor Pressure

Turgor pressure is a crucial parameter (Deri Tomos et al. 1989); yet, its measurement is technically challenging. A series of different methods have been proposed and used, but there have been only a handful of successful experiments on growing organs. The first methods were based on finding the point at which the turgor pressure in the cell is balanced by the pressure in the mounting media. Above a certain threshold, the turgor pressure does not act on the cell wall and the cell is plasmolyzed. It is important that the osmolyte (the ion used in the medium to compete with the cellular ionic concentration) cannot be internalized by the cell, and that water can flow freely out of the tissue (Falk et al. 1958; Nilsson et al. 1958; Stadelmann 1984).

Microscopy observation can be used to observe when the ionic activity of the external solution matches the cell. A classic classroom experiment is often performed on naturally colored cells such as red onion or flower petals. The limitation of this method is the field of view of the microscope. For a full tissue, one can use vibration to determine the plasmolysis point. This technique relies on the fact that the vibration properties of a tissue are related to its rigidity, and the rigidity depends on the turgor pressure (Virgin 1955). The rigidity drops with a drop in pressure until plasmolysis is reached. At this point, the rigidity is not sensitive to plasmolysis and depends only on the cell wall elasticity.

Another approach is to measure the pressure directly by puncturing the cell with a tube. This method works for big cells, but not for the very small cells important for growing tissue (Green 1968; Green et al. 1971; Büchner et al. 1981). The most complete measurement was done on the root, but the authors could not detect any differences in the turgor pressure along the elongating roots. This indicates that, so far, there is no evidence to support the action of turgor pressure on the variability of growth rate observed within the organ.

**Summary** The tools available for measuring turgor pressure are not precise enough to measure single-cell turgor pressure in the early stages of organ formation. This is especially true for the model plant *Arabidopsis thaliana*, which has particularly small cells.

## 7.5 Cell Wall Rheology

### *7.5.1 Definition*

Rheology studies the deformation and flow of matter; here we review the rheology of a particular hydrogel, the cell wall. Like any hydrogel, the mechanical properties of the cell wall change with the amount of water it contains. Importantly, once dehydrated, the cell wall has irreversibly lost its original mechanical properties.

The mechanical properties of a hydrogel depend on the ionic composition of the solution. Ions influence water activity (i.e., cell wall hydration) through their affinity to water. Monovalent ions intercalate into the gel and affect the distance between the polymers working as plasticizers. In contrast, divalent ions can create bonds between the charged molecules of the polymer mesh. For more details, please refer to Sect. 6.2 on pectin. Thus, the mechanical properties of the primary cell wall can only be determined on the fresh, intact cell wall with a protocol that does not change the ion composition.

How do we measure the mechanical properties? One method is to deform the sample and record the force required over time. Alternatively, a constant force can be applied and the change in shape of the sample recorded over time. Several parameters can be measured in this way, but depend on the type of deformation observed. If the deformation is reversible, elasticity is measured (the cell wall regains its original shape after the force has been removed). The time taken to come back to its original shape is a measure of the viscoelasticity. If extension is irreversible, the viscosity of the material is measured (Braybrook et al. 2012). To measure the change in shape indirectly, one looks at the indentation depth or uses fluorescent probes (Kim et al. 2015).

In plant biophysics, the majority of mechanical measurements are designed to measure the growth capacity of the tissue; thus, a different rheological property of the cell wall is measured, the creep.

### *7.5.2 Creep*

The definition of creep is inconsistent in the literature. In general, creep refers to the growth capacity of the tissue. It could be thought as the *m* factor in the Lockhart equation (Taiz 1984). If growth occurs mainly as a result of rearrangement of the cell wall network, it can be measured as the energy required to stretch the cell wall (Keegstra et al. 1973). Many components involved in loosening of the cell wall have been characterized with this method. One of the founding fathers of this type of measurement is Paul Green, who worked on giant cells from Characeae green algae (Green 1976). He measured the relative importance of turgor pressure, elasticity, and creep in growth of the cell wall, thanks to measurement of extension at a subcellular resolution and the extension capacity. Green always took a critical view of creep measurement and its inability to separate the contributions of rearrangement of the cell wall and cell wall synthesis (Green and Cummins 1974).

Recently, cell wall rearrangement during creep has been observed thanks to the use of atomic force microscopy (AFM). The studies demonstrated that cell wall rearrangement, at least in the epidermis, is associated with elongation of the tissue and is reflected in the creep experiments (Zhang et al. 2017).

### 7.5.3 Other Measurements of Cell Wall Rheology

Measurement of the elasticity, viscoelasticity, and viscosity in living tissue using a nanoindenter has recently been developed. Surprisingly, elasticity (reversible deformation of the cell wall) was correlated with growth and not viscosity or viscoelasticity (Peaucelle et al. 2015). This is paradoxical because elasticity is a reversible deformation, whereas growth is an irreversible process. At first glance, the finding is also in opposition to creep experiments that put cell wall remodeling at the heart of the growth process. This can be explained if elasticity correlates with growth through control of cell wall synthesis and not cell wall remodeling. In other words, the synthesis of new material is linked to the elasticity of the cell wall. This correlation was first demonstrated on pollen tubes: Local changes in cell wall elasticity correlated with the position of exocytosis of cell wall components at the tip of a cell. This process was observed to involve cytoplasmic calcium signaling coupled with deformation-sensitive calcium channels (Fayant et al. 2010).

**Summary** Creep experiments directly measure the ability of the cell wall to rearrange in association with growth. Elasticity of the cell wall relates to growth in manner that could be related to cell wall synthesis (but has yet to be determined). Therefore, two independent growth processes could relate to different cell wall rheological properties.

## 7.6 Organogenesis and Polar Growth of Tissue: Contribution of the Cell Wall Component

The turgor pressure that drives cell expansion is isotropic. If it was the only parameter controlling growth, turgor pressure could lead to homogenous elongation (i.e., a sphere). Then, plants would look like a drawing of La Gioconda by Botero (Fig. 7.3). Somehow, this isotropic force is transformed into anisotropic orientated growth. Which component of the cell is responsible? A good candidate is cellulose (Green 1980).

### 7.6.1 Cellulose

Determination of the structure of cellulose was a long and difficult path. It took 30 years from the first chemical isolation of cellulose to determination of its polymer structure. From the start, cellulose was considered to be the load-bearing component of the cell wall and responsible for anisotropic growth.

The basic idea is that bundles of cellulose fibrils build up an orientated network surrounding the cell and block expansion in one direction. We could compare it to

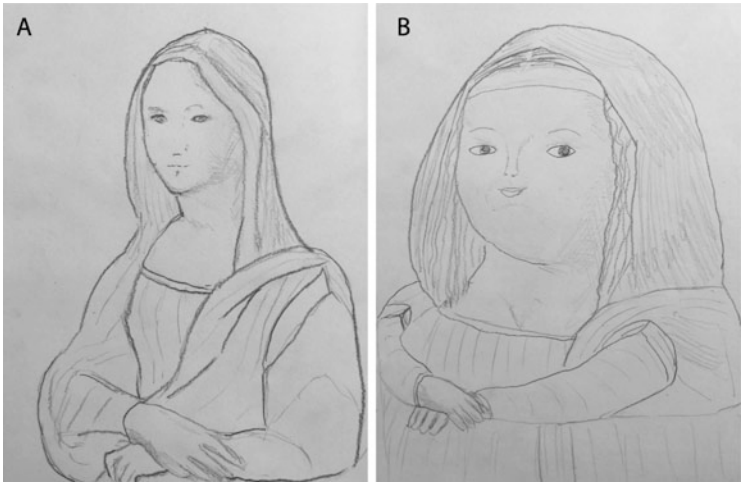


**Fig. 7.3** Isotropic growth illustrated by (**a**) representation of La Gioconda of Leonardo da Vinci and (**b**) La Gioconda of Botero

the metal rings around a barrel that prevent it from opening up. Electron microscopy data and cell wall optical imaging support this theory. A series of brilliant images showed orientated microtubules not exactly perpendicular to the cell but organized in sheets like the laminated structure of wood (Fig. 7.4). At the same time, Paul Green observed in giant algae cells that the cellulose fibrils were orientated in a looser way, in a network (Green 1960; Gertel and Green 1977). These two publications mark the point when the scientific community divided into two camps. The first theory supports the laminated organization of cellulose and suggests that orientation of the fibrils in a sheet is stable during growth. Loosening between lamellae leads to progressive reorientation of the whole sheet. In contrast, the network theory, following Green's observations, suggests that the latest microtubules are deposited in an orientated way, but that growth modifies their orientation and distorts the network. In this theory, only the most recently synthesized cellulose fibrils control anisotropic orientation (Marga et al. 2005). Recent observation by Cosgrove and colleagues (Zhang et al. 2017) of a multinetwork structure and its reorientation supports the network concept. In fact, both visions could be right: The multinetwork structure was observed in the external cell wall of epidermal cells, whereas the laminated structure was mostly in the internal cell wall. Thus, the two concepts of cell wall structure might simply relate to two different types of cell wall.

The key role of cellulose in anisotropic growth was most strikingly demonstrated by the swelling of plants following chemical treatment affecting cellulose or microtubule synthesis and by the phenotype of a mutation affecting cellulose synthase (Ledbetter and Porter 1963; Heath 1974; Mueller and Brown 1982). The similarity of this phenotype to the result of inhibition of microtubule synthesis led to the idea that the orientation of microtubules is generated by the orientation of

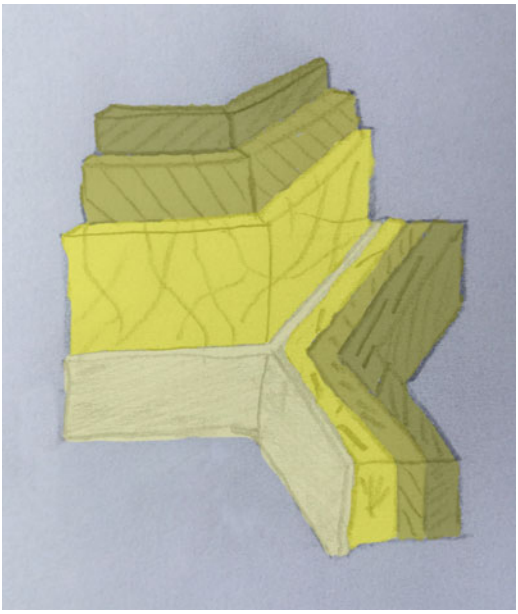

**Fig. 7.4** Scheme of a laminated cell wall

cellulose (Heath 1974). This concept is supported by the observation that cellulose synthase and microtubules are found in close proximity. A commonly used model states that cellulose synthase polymerizes cellulose directly in the plasma membrane following orientation of the microtubules and is supported by microscopy observations. The microtubule orientation then leads to mechanical anisotropy in the cell wall and anisotropic expansion of the cells.

To complicate this picture, recent work (Peaucelle et al. 2015) has shown that treatments affecting cellulose synthase or microtubule orientation also affect another component of the cell wall, pectin.

### *7.6.2 Pectin*

Pectin forms a fine meshed network surrounding the other components of the cell wall. There are several chemically different components of pectin, but here we focus on the homogalacturonans. This component is a polymer of galacturonan sugar, which presents a lateral carboxyl group that can be methylated or not. In the 1980s, the 3D structure of the two polymers was predicted (Morris et al. 1982). Methylated pectin was predicted to form a very compact structure, with proton-stabilized interaction on the methylated carboxyl (Grant et al. 1973).

Demethylated pectins were anticipated to generate a more hydrated and less packed structure stabilized by calcium electronic interactions through demethylated carboxyl groups. This model was named the egg box structure, where the stability

of the conformation would be archived for at least nine successive demethylated homogalacturonans. At first, the calcium bonds found in demethylated pectin were thought to generate strong links in the cell wall and thus limit cell wall remodeling and creep. It was suggested that they slowed down growth. Interactions between methylated pectin were ignored, except in the food industry. The vision of demethylated pectin linked to a rigid cell wall was first challenged when pectin demethylation was shown to lead to organ formation and reduction in cell wall elasticity in the meristem (Peaucelle et al. 2011).

Later, it was found that the anisotropic changes in pectin methylation are required for polarized elongation of the hypocotyl and are associated with a reduction in cell wall elasticity (Peaucelle et al. 2015). This finding led to the proposal of a two-step process for anisotropic elongation of the tissue: Antipodal changes in cell wall elasticity caused by changes in pectin methylation lead to a tenfold anisotropic elongation. This anisotropic growth is followed by alignment of microtubules and cellulose microfibers. Thus, cellulose microfibers are needed for further anisotropic elongation, which can achieve 100- and even 1000-fold anisotropic elongation. Furthermore, these two components interact, as demonstrated by treatments affecting microtubule and microfibril orientation, which also affected the pectin methylation pattern (Peaucelle et al. 2015).

**Summary** Polar elongation is a two-step process: First, a change in pectin methylation leads to a change in cell wall elasticity, followed by cell polarity (cell mechanical asymmetry). Microtubules reorient along the elongation axes, leading to orientated cellulose synthesis. This generates cell wall anisotropy.

### *7.6.3 Xyloglucans*

Xyloglucans are components of hemicellulose that have attracted a lot of attention since their strong interaction with cellulose was described. Models predict that reducing the amount of xyloglucans could increase creep by decreasing cellulose microfibril cohesion and helping local rearrangement of the cell wall.

The enzymes that control the structure of the xyloglucan network have been predicted. The genes coding for these proteins are expressed in a tight developmental pattern and are present in sites with strong elongation (Antosiewicz et al. 1997). Unfortunately, multiple mutations in these genes do not present an obvious growth defect phenotype. Are xyloglucans without a function? Certainly not. We have seen that there are multiple mechanisms controlling growth; therefore, it is likely that the absence of xyloglucan remodeling is compensated (Cosgrove 2016).

### 7.6.4 *Expansins*

Expansins form a family of cell wall proteins. Their importance in growth was demonstrated when purified expansin proteins were shown to accelerate growth in some tissues (Fleming et al. 1997). They are the only known proteins to promote creep in vitro (Cosgrove 1998; Shieh and Cosgrove 1998). There are two isoforms present in a multigene family found throughout the plant kingdom (Cosgrove 2015). The first isoform interacts with cellulose and the second (found in grasses) interacts with glucuronoarabinoxylan, a grass-specific carbohydrate. Interestingly, only specific cells are sensitive to expansins. This suggests that not all cell walls can respond to expansin, demonstrating multilevel control (McQueen-Mason and Cosgrove 1995).

**Summary** Cell wall chemical components have redundant functions in cell wall mechanical properties and growth. This chemistry is very dynamic and is under the control of complex signaling networks that are still to be described. So far, we have seen only the tip of the iceberg of this chemical complexity.

## 7.7 Input from Growth Measurements

Understanding plant cell wall mechanics and its link to cell wall chemistry is only one part of the problem. It is also important to undertake detailed quantitative measurement of the growth process, in particular plant growth-induced motion.

Observation of plant motion has been at the heart of scientific debate for a long time. First reported in 400 BC, it was also discussed in Hook's famous publication, which coined the word "cell." Growth-related motion, in particular circumnutation, fascinated Charles Darwin (Darwin 1880). The first movie of a growing plant dates from the end of the nineteenth century, yet quantification of growth is still difficult because it occurs in three dimensions. Until now, only 2D growth in response to gravity has been fully described (Erickson 1976).

Those early films revealed that plants adapt their shape to external stimuli such as light and gravity. These growth movements are named phototropism and gravitropism, respectively. There also exist lesser known growth movements such as ototropism, also named proprioception (Bastien et al. 2013). Proprioception means that plants are able to sense their own shape and control tissue growth so that they stay straight. The shape of *Arabidopsis* grown in microgravity at the international space station illustrates proprioception very well. Plants grown in space are almost identical to control plants grown on Earth (Link et al. 2003, 2014). Study of gravitropism in Earth-grown plants has led to the same conclusion. These exciting results reinforce the crucial importance of the feedback loop between growth mechanics and tissue structure, not only at the subcellular level but also at the whole organ and organism level.

Another fascinating thing about plant motion linked to proprioception is oscillatory movement, which reveals complex regulatory networks of growth acting at different time scales. It also explains the redundant functions and parallel growth processes we have discussed so far.

## 7.8 About the Regulatory Network

The next step is to explore the regulatory networks involved in growth. The study of signaling network in plants is described in other chapters of this book; here, we briefly discuss two aspects. The auxin regulatory network is the most studied aspect. The plant hormone auxin was isolated thanks to its capacity to promote growth. The growth induction capacity of auxin was rapidly associated with the acid form of the molecule. It was proposed that auxin promotes growth through acidification of the cell wall, leading to cell wall rearrangement. This model was rapidly confirmed by the observation that the expanding cell wall has a low pH (Tepfer and Cleland 1979). Intriguing information about the auxin growth network was obtained from study of the meristem and generation of the phyllotactic pattern. Since the work of Stephane Douady and Yves Couder, we have known that generation of the phyllotactic pattern requires a dynamic feedback loop between inhibitory and activating signals in the meristem (Douady and Couder 1992). Isolation of the *pin1* mutant and the development of fluorescent reporters enabled the discovery that auxin is the activator molecule necessary and sufficient for induction of organ formation (Okada et al. 1991). The dynamics of the auxin transporter system in the meristem depletes auxin in the areas surrounding new organs and thus inhibits formation of new primordia nearby (de Reuille et al. 2006). The authors suggested that the dynamics of the structure was generated by the auxin concentration itself.

Recently, auxin was shown to induce pectin demethylation in the primordia and that this change was necessary and sufficient for organ formation (Braybrook and Peaucelle 2013). Intriguingly, pectin demethylation is also necessary and sufficient for auxin-induced growth, suggesting that auxin acidity is not sufficient for organ growth and that the acidification of the cell wall commonly associated with growth could instead be attributed to acidification by carboxyl groups formed during pectin demethylation. Regulation of polar auxin transport was also questioned; it could not simply be controlled by auxin concentration because the changes in cell wall chemistry lead to destabilization of polar auxin transport. In parallel, cell ablation experiments in the meristem showed that, like microtubules, auxin polar transport responds to mechanical stimulus (Hamant et al. 2011). These results suggest that polar auxin transport is at least partially under the control of mechanical constraints arising from the differential cell wall elasticity of the growing organ. This feedback loop is at the heart of organ formation. How exactly this feedback is generated is still to be discovered; it could be via chemical or mechanical signals.

How mechanical clues from the cellular environment can be synthetized and transduced to the cell is also an important future research area (Wolf et al. 2012). One important component of this regulation is the transmembrane kinase receptor (THESEUS and FERONIA are the most studied receptors). It is possible that the extracellular domain of this protein can sense the chemical/mechanical stress of the cell wall and feedback through a kinase cascade to the nucleus and affect gene transcription. The beauty of the kinase-signaling cascade is its integrative capacity (for more information, read about the regulatory kinases in animal cell cycles). If the regulatory system of plants is as complicated as that described for mammalian cells, it could be decades before we can grasp all the subtleties of these regulatory networks.

## 7.9 Conclusions and Perspectives

Clearly, we are far from understanding the mechanics of plant growth. Yet, we are gaining new insights from all directions at an incredible pace. The precise description of several of the key elements regulating growth forms the basis for study of the regulatory network. However, part of the process is still invisible. A complete understanding of the process is currently out of our reach, either because of its complexity or because we lack a crucial aspect. We still do not have a satisfactory answer to our original question: How does the cell wall expand without the cell bursting? New technology and thinking out of the box will certainly help to solve this puzzle.

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# Chapter 8
# Apoplastic Barriers: Their Structure and Function from a Historical Perspective

**Aleš Soukup and Edita Tylová**

**Abstract** The multicellular plant body is a complex structure that is internally organized into organs and tissues specialized for particular functions. The outer boundary of a plant body is delimited by the epidermis, and its specific modifications allow controlled material exchange with the surrounding environment. The inner space of a plant body is subdivided into functional domains. Such division is known to take place in both the symplastic and apoplastic spaces of organs and tissues. The apoplast is composed mostly of intercellular spaces and porous cell walls. It surrounds the symplast, which is bordered by the plasma membrane. The internal subdivision of apoplastic space is carried out by so-called apoplastic barriers, which are cell layers with modified cell walls where lowered porosity decreases the passive flow of solutes, water, gasses, and regulatory molecules. There is a well-established role of the endodermis in the function of the root in vascular plants. However, the root endodermis is not the only "barrier" essential for plant function and development. Similar barriers are known to be present in stems, leaves, and the root periphery. This review focuses on the historical course of our understanding of the development, structure, and function of these protective layers.

## 8.1 First Discoveries

The first known systematic works related to apoplastic barriers are probably those of Johann Xaver Robert Caspary (1818–1887), a German botanist who dedicated most of his efforts to aquatic plants and vascular tissues. However, even Caspary mentioned French botanist Jules Émile Planchon (Planchon and Van Houtte 1851), who observed a specific cell layer surrounding a central system of vascular bundles in the roots of *Victoria regia*; these cells contained dark dots in the radial cell walls (Fig. 8.1).

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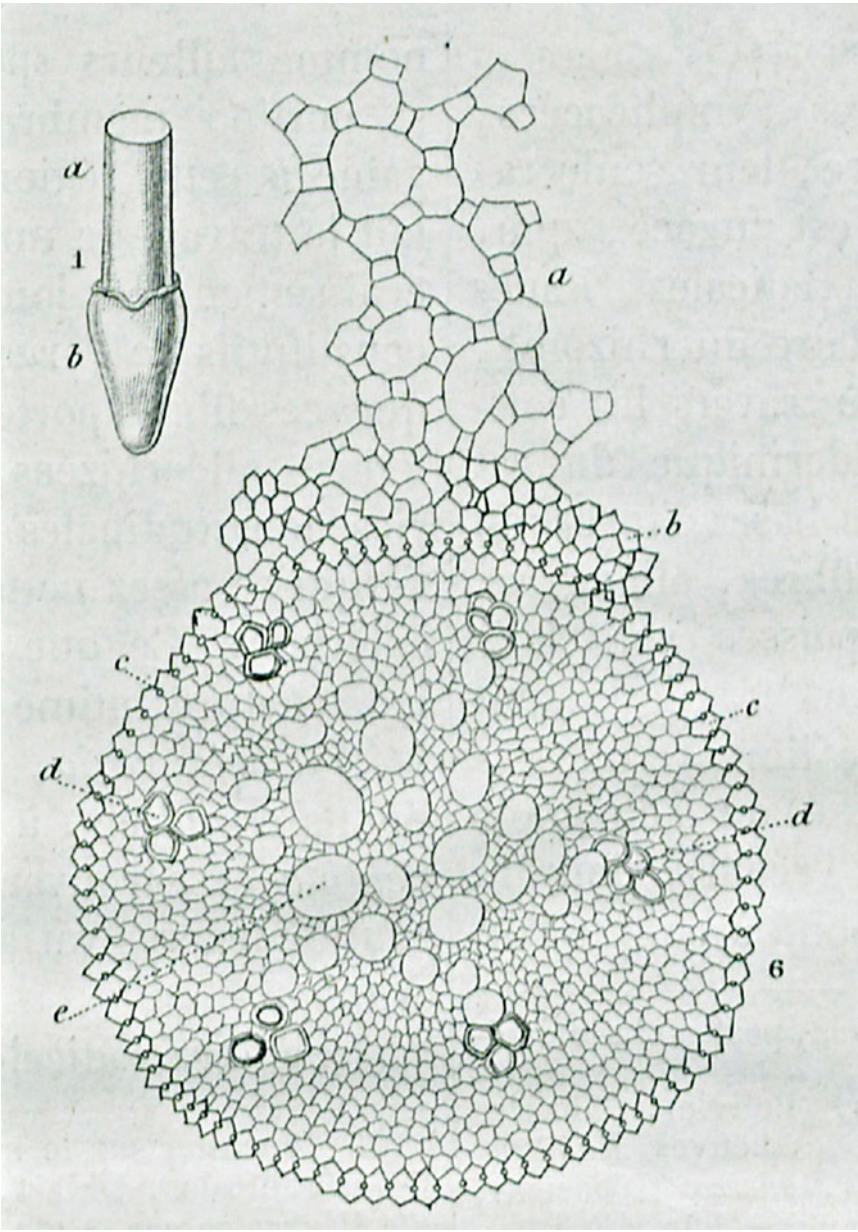


**Fig. 8.1** Drawing of *Victoria regia* root cross-section from Planchon's publication (Planchon and Van Houtte 1851) demonstrating dark spots "pores" in the endodermis. Description as in original text: *a* lacunar tissue, *b* internal non-lacunar tissue, *c* ring of vessels marked with a row of punctuations on their contact faces, *d* two of the vascular bundles of first formation, already empty, and contiguous with each other, *e* one of the vessels of second formation: their internal content is not visible, therefore they might be considered as gaps

However, the structures did not attract Planchon sufficiently to follow the theme in his later work.

Caspary described a continuous cylinder of tightly packed cells surrounding vascular bundles, which attracted his attention because of the specific shape and presence of dark dots in the radial cell walls of the shoot of *Elodea Canadensis* (Caspary 1858) and later in other species and organs. He described this histologically distinct cell layer as "*Schutzscheide*" (protective layer), thus opening an interesting topic that attracted his contemporaries and still remains an interesting field of research.

Searching for the nature of the dark dots, Caspary examined the roots, stems, and leaves of various species. Studying *Adoxa moschatelina*, he negated his original opinion that the dots might be related to tiny pores in the radial cell wall and understood that they are visible because of local folding of the cell wall (also observed in *Ficaria ranunculoides* (Fig. 8.2), *Elodea canadensis*, *Brasena peltata*, and *Charlwoodia rubra*). The folding gradually disappeared during later thickening of endodermal cell walls, and Caspary attributed the loss to subsequent cell elongation (Caspary 1865). The presence of corrugated strips was considered by some authors to be an artifact of sectioning that resulted from different tensions within the middle part and the rest of the radial cell walls (for a review, see Schwendener 1883). This opinion was contradicted by others, who demonstrated the presence of these structures in intact cells (Wisselingh 1886).

Caspary also reported differential staining of the radial cell walls of Schutzscheide by iodine and the resistance of these layers to chemical digestion, valuable techniques used at that time to gain a better understanding of the properties of cell walls. He found a 4–8 μm wide central wavy band (later called the Casparian band) to be particularly resistant. Caspary treated cells of the *Schutzscheide* with sulfuric acid and Schultz's maceration solution (strong nitric acid and potassium chlorate). Because of its resistance and staining properties, he excluded cellulose as the principal material of the strip and asked whether it was a "wood-like" or "cork-like" modification of the cell wall. He emphasized in his comments that no specific test had been available to distinguish clearly between those two types of cell wall modifications. Digestion-resistant material formed a continuous cylinder surrounding the remains of vascular tissue, being "very closely linked forming thus an annular skin" (Caspary 1865). His detailed observation also indicated disappearance of the dark spots during later deposition of thickened cell wall. He mentioned the lack of pits (tiny pores) in the thickened cell walls in some species and questioned the extent to which such a layer of closely packed cells with thickened internal tangential and radial walls would allow gas and solution transport. He also expected that such a cell cylinder would protect the inner tissues from the influence of outer conditions. However, no experimental support was available at that time and the functional features of the endodermis and similar barriers were only elaborated much later.

Caspary described in detail the gradual development of the cells of the endodermis as follows:

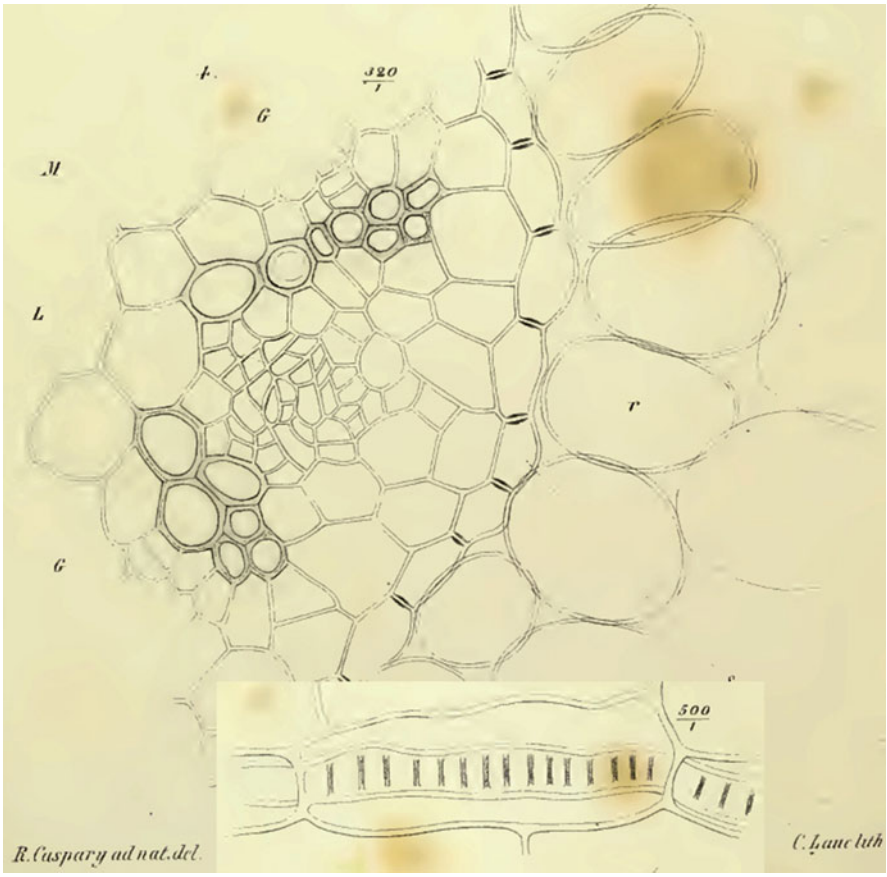


**Fig. 8.2** Cross-section of *Ficaria ranunculoides* adventitious root from Caspary's publication (Caspary 1865). Description as in original text: Part of cross section of the vascular bundles system after caustic potash treatment. *S Schutzscheide*, *G* vascular bundles, *M* pith, *r* cortex, *L* phloem bundles. The *inlay* shows details of Schutzscheide cell on the longitudinal section mount in water

1. Dark spots are formed during the early stages of development as a result of corrugation of the middle or offset part of the radial cell walls of the *Schutzscheide*.
2. The corrugated part of the cell wall becomes wider and soon covers almost the entire width of the radial wall.
3. The corrugated part of the wall becomes modified in a "woody" manner, similarly to cuticle or cork.
4. In many species, the dark dots are not visible once the secondary deposits are built up on the inner tangential and radial cell walls and to lesser extent on the outer tangential cell wall.

5. The endodermis might have no pores (pits) in the thickened cell walls (at least in *Dracaena*).
6. The *Schutzscheide* cells form a firmly closed tube without intercellular spaces, enclosing the tissue that they surround.

Caspary also noticed some cases of later development connected with deposition of lignified cell wall in the root endodermis of *Cyperus papyrus*, *Scirpus lacustris*, *Phragmites communis*, and *Dracaena*, and in the stamens of *Potamogeton*. Caspary did not distinguish the suberin lamella as did later authors, but included this specific layer in the primary cell wall. The standard sequence of endodermal development through the three successive stages of Casparian band formation (primary endodermis), deposition of suberin lamella (secondary endodermis), and thickening of the cell wall (tertiary endodermis) was established later by Kroemer (1903) and Schoute (1903).

Caspary speculated about the histological origin of *Schutzscheide* (in concurrent literature also known as "vagina tutelaris," protective sheath) and came to conclusion that this layer originates in the cortex parenchyma. This opinion was not shared with his contemporaries. Caspary especially mentioned Karsten, who considered the *Schutzscheide* to originate in the cambium, according to its position between vascular bundles and cortex (Caspary 1858; Karsten 1849). Besides Karsten, Schacht and Sanio also considered the endodermis to originate in the cambium (*Cambiumschicht*), which produces the radial arrangement of stem tissues and allows for later radial growth. Caspary disagreed with those authors and explained that a local cambium layer (*Cambiummantel*) does not form primary stem tissues, but that cells of the shoot tip (terminal bud) act as the source of maternal cells for primary tissues (Caspary 1865); cambium producing later/secondary tissues should be located within vascular tissues, as indicated by work of Nägeli (1858).

Caspary's term "*Schutzscheide*" was later substituted with "endodermis," most probably because of De Barry's use of the word in his influential textbook (De Barry 1884). The earliest usage of the term "endodermis" can be traced back to Oudemans (1861), who used it to label the inner skin of the aerial roots of orchids in his description of their sulfuric acid-resistant layer underlying the velamen (exodermis in current terminology). De Barry (1884) adopted this term, but used it in a wider sense for description:

"The endodermis is a sheath consisting in all cases of one single layer of cells. It should also be observed here, that it lies as a rule at the limit between masses of parenchyma and other systems of tissue, especially vascular bundles, and is then to be recognized both by its development and its mature properties, as the layer of the parenchymatous mass bordering on the unlike parts."

Such a definition clearly expects any apoplastic barrier to have physiological functions and connects those with their typical properties. For both De Barry and Caspary, the endodermis consists of radially flattened cells with wavy radial cell walls resistant to sulfuric acid (lignified/suberized) that are tightly packed without intercellular spaces into a hollow cylinder. Their tangential walls are rarely and delicately pitted. Such a description is histological, without definition of position

within the organ, and describes the endodermis well; but, it also describes the exodermis as currently recognized (Enstone et al. 2003; Geldner 2013; Hose et al. 2001). In fact, the terms inner and outer endodermis were used by some authors in the sense of endodermis and exodermis, in addition to other terms such as *Strangscheiden*, *Gefässbündelscheiden*, *verholzter Verdickungsring*, *Rindenscheiden*, etc. (von Höhnel 1878). Similarly, the definition of von Höhnel (1878) seems to reflect the most common features of endodermis (in a wider sense of meaning), stating that in his understanding the endodermis is a single-layered structure consisting of seamlessly interconnected living cells, the walls of which show the construction of cork cell walls. Where it is possible that individual cells or cell strips within the cylinder are not corked.

## 8.2 Modifications of Cell Walls at Apoplastic Barriers

The modification of cell walls of apoplastic barriers (endodermis and exodermis) develops gradually during differentiation. There are often three principal stages of cell wall modification, which might vary according to plant species or the position of the apoplastic barrier within the plant body. The three stages are formation of Casparian bands, formation of suberin lamellae, and secondary cell wall deposition (Enstone et al. 2003; Geldner 2013). There is a gradient from younger to older parts related to cell differentiation, but individual cells can differ from their neighbors, even with the same position along the axis. Nikolai (1865) studied the roots of *Polygonatum multiflorum* and several orchids and observed that cells of the endodermis in the vicinity of xylem vessels exhibit delayed cell wall thickening. The tangential variation within the layer was noticed by von Höhnel (1878) and the presence of passage cells (*Durchlaszellen*) abutting xylem strands, which do not have suberin lamellae, was observed by Schwendener (1883). Variation in cell wall modifications within the endodermis and analyses that do not take into consideration primary and secondary stages of endodermal development have resulted in many inconsistencies in the published data and complicate subsequent interpretation of cell wall chemistry.

The Casparian band is the only cell wall modification present in the primary developmental stage of endodermis. As summarized by von Guttenberg (1940), the band almost always tests positive in lignin-detecting reactions (e.g., HCl-phloroglucinol, Mäule's reaction, aniline sulfate) and also in many cases for lipid compounds. The debate over whether modification of the endodermis and similar structures is suberized (*verkorkt*) or lignified (*verholzt*) runs through endodermis research from the very beginning (Caspary 1865). This is partly due to the lack of precise definitions of these terms and the absence of suitable detection methods in the nineteenth century. The absence of specific histological approaches prevented such a conclusion from being made at that time (von Höhnel 1878).

Suberization (*verkorkung*) was recognized as a general feature of the endodermis and similar apoplastic barriers, and its relationship with physiological function was

suggested (De Barry 1884; Oudemans 1861; von Höhnel 1878). In analogy with other structures forming a physiological sheath/barrier (epidermis, cork, and endodermal cells), suberization was recognized to be connected with the presence of lipid material in the cell walls, which could be extracted from the cell walls by potash lye treatment and then dissolved in polar solvents (von Höhnel 1878). However, one should realize that Höhnel's work did not reflect the developmental sequence and, thus, he tested mostly secondary endodermis containing suberin lamellae. Therefore, his conclusion and demonstration of lipids in the endodermis should be seen from this perspective and not as directly connected with the Casparian band. There are reports indicating that suberized strips in the radial walls occur in the primary stage of endodermis formation, but they are not general (De Barry 1884; Wisselingh 1886). Tertiary development starts with the deposition of a frequently massive secondary cell wall. Russow (1872) distinguished between O-sheaths, with uniform thickening of all walls, and C-sheaths, with internal horseshoe-shaped thickening (Fig. 8.3). An interesting early observation was that,

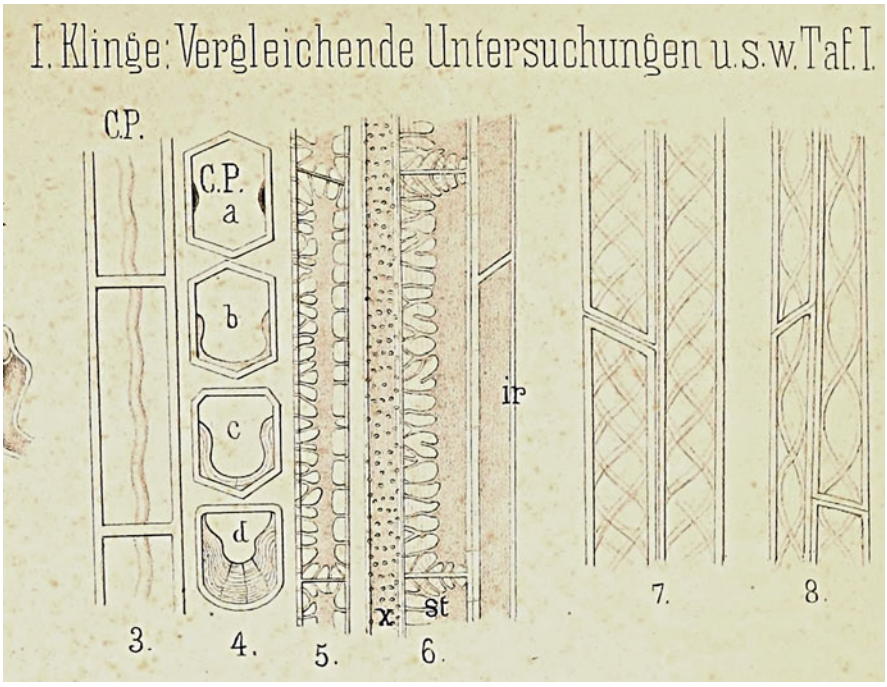


**Fig. 8.3** Description of drawing as in original text (Klinge 1879): *3 Milium effusum*, longitudinal section of *Schutzscheide* cell with thickening of Caspary dot. *4 Stipa pennata*, progressive development of tertiary endodermis: *a* pure Schutscheide cell; *C.P.* Caspary's point; *d* fully developed *Schutzscheide* cell with layered cell wall and pits. *5 Cyperus Ginge*, longitudinal section of mechanical cell of *Schutzscheide*. *6 Phalaris arundinacea*, longitudinal section of *Schutzscheide* cell (*St*). The thickening with marked pits and obvious layering is directed only toward the inside of the vascular bundles; *x* xylem vessel; *ir* the innermost cortical cell. *6 Saccharum cylindricum*, striped cells of the external cortex. *7 Saccharum cylindricum*, striped cells of the pith

in *Poaceae*, the inner secondary cell wall of the tertiary stage might be silicified (Klinge 1879; Kroemer 1903). Kroemer (1903) observed that silicification extended from the pits of the tertiary cell wall deposits.

We will now have a more detailed look at suberin, which is commonly connected with the development of apoplastic barriers. The term "suberin" was, according to von Höhnel (1878), used for the first time by Chevreul (1815) in his work dedicated to the analysis of cork. Extraction in hot water and alcohol yielded a residuum that he considered to be the basis of the cork cell wall and named it suberin. However, opinions about the nature of suberin at that time were far from consistent; even then, authors were aware that Chevreul's residuum contained cellulose and other components. The fact that the cellulosic wall is part of the cork material, or that suberin modifies the cellulosic cell wall, was demonstrated by Mohl (1847) during his microchemical tests. Some authors (e.g., Sachs, Dippel) considered that suberin is in fact modified cellulose, whereas others (Weisner, Haberland, Schulze) considered suberin to be a specific compound on its own (for a review see von Höhnel 1878). Therefore, the early definition and concept of suberin is rather loose, without good chemical definition. The opinion that lignin and suberin (cuticular substance) are cellulose impregnated by various "impurities" was rather common in the second half of nineteenth century. Such a conclusion is rational in the light of later discoveries, which used various macerations, extractions, and melting point experiments to identify the lipid material (suberogenic acids) impregnating cuticle, cork, and endodermis (for an extensive review, see Kroemer 1903).

Wisselingh (1886) recognized the variation among lipidic cell wall components from different sources (cuticle, cork, endodermis) according to their extractability and melting point. Similarly, Gilson (1890) found it difficult to reconcile the insolubility of suberin in nonpolar solvents and therefore expected polymerization and linkages to hold the lipids within the cell wall. Because the chemical nature of the material was uncertain and variable, Kroemer (1903) decided to distinguish the lipid material within the cell wall according to its position: cutin is present in epidermis, and suberin in cork and endodermis. Many early authors described cell walls as "cutinized" to indicate the presence of lipid compounds in the endodermis. The material of suberin lamellae shares features previously identified by von Höhnel (1878): insolubility in sulfuric acid and Eau de Javelle (sodium hypochlorite), low solubility in cold 50% chromic acid, and brown coloration after treatment with chlor-zinc-iodine solution (Zimmermann 1892).

The visualization of suberin lamellae after digestion was achieved with staining. The most significant further development was introduction of Sudan Red III dissolved in alcohol (Buscalioni 1898), which is a dye rather specific for lipidic suberin. This solution was further optimized by Kroemer (1903) to the following recipe: 0.01 g of Sudan III in 7 mL of 96% ethanol and addition of 5 mL of glycerin. Currently, the best modification of Sudan staining is that of Brundrett et al. (1991) using PEG as solvent. Kroemer (1903) reported Sudan staining of Casparian bands to be weak or negligible compared with that of cuticle or suberin lamellae and

suggested that staining might be connected with membrane associated with the Casparian band. Other less specific pigments dyeing both lignified and suberized cell walls were also used, as summarized by Zimmermann (1892). These included ammonia-fuchsin according to van Tiegliem, chlorophyll according to von Correns, and Alkannin.

Lignin or lignin-like material, which is very typical for Casparian bands during the primary stage of development, was recorded by early authors such as Kroemer (1903), primarily using HCl-phloroglucinol staining (Zimmermann 1892). Interestingly, this method is not efficient for the earliest stages of development when Casparian band position is detectable only by cell wall undulation, as mentioned for *Zea mays* (Kroemer 1903). To distinguish between lignified and suberized material, staining was combined with sodium hypochlorite digestion to remove lignified material (Kroemer 1903). Hot Eau de Javelle dissolved lignin, and prolonged treatment stopped HCl-phloroglucinol staining (Kroemer 1903). The Casparian band was not destroyed in Eau de Javelle or concentrated sulfuric acid, but did dissolve after prolonged treatment with chromic acid (Kroemer 1903), which means that it is not "only" lignin that composes the structure. In cold potassium hydroxide, which also extracts lignin, the Casparian band turns a pale yellowish color that gradually disappears after heating. Although the Caspary band finally appears colorless, it remains strongly refractive compared with the surrounding cell wall (Kroemer 1903). It is interesting that Kroemer noticed that treatment with Eau de Javelle resulted in many fine Sudan Red stained lipid globules associated with the Casparian band. His description of changes in solubility of the Casparian band during sequential digestion provides a nice indication of differences in composition of the Casparian band and surrounding cell wall. In sulfuric acid, the Casparian band, modified by the preceding action of Eau de Javelle (a strong oxidative and delignifying agent), dissolves completely. Even dilute sulfuric acid can suffice under certain circumstances, whereas the cellulosic cell walls outside the Casparian bands only swell strongly without dissolving (Kroemer 1903).

Such observations might suggest that the polyaromatic domain of suberin, which is detected as (or in fact is?) a lignin material, is the principal scaffold of Casparian bands. This model fits well with current knowledge and models describing suberin (Franke and Schreiber 2007; Kolattukudy 1980) as a polyester of long-chain aliphatic acids, alcohols, and glycerol. This acylglycerol lipid moiety is esterified to ferulates via ω-hydroxyacids (Graca and Pereira 2000), forming aliphatic–aromatic linkages that are considered important for arrangement of suberization within the cell wall (Graca 2015). The extent to which this polyphenolic domain of suberin is similar to or different from conventional lignin is still controversial (Graca 2015). Some authors consider only the lipid domain to be "true" suberin because of chemical, structural, and (in suberin lamellae) spatial separation of the aromatic domains (Graca 2015). Should we consider therefore the Casparian bands as only lignified?

Tight connection of the plasmalemma (plasma membrane) and cell wall is another essential aspect of Casparian bands. In fact, the resulting band plasmolysis

was frequently used to confirm their presence. It is interesting that the mechanism of this plasma membrane–cell wall connection is still unresolved. Tight association of plasma membrane and cell wall in the region of the Casparian band is the crucial feature that allows the restriction of apoplastic transport and direction of material flow toward the symplast. This tight link was recorded as early as the end of the nineteenth century (Kroemer 1903; Wisselingh 1886) and the term "band plasmolysis" established for the specific pattern of plasmolysis (Behrisch 1926) typical for the primary endodermis. Band plasmolysis was later documented in both the endodermis and exodermis of various species (Bryant 1934; Enstone and Peterson 1997; Haas and Carothers 1975; Haas et al. 1976; Karahara et al. 2004; Lehmann et al. 2000; Ma and Peterson 2001; Peterson and Emanuel 1983; Schnee 1936; Zankowski et al. 1987). The mechanism of the association, however, remains unclear. Scott (1963) assumed that the high frequency of plasmodesmata might by the reason for the pattern of adhesion, but this idea does not correspond with the obvious lack of plasmodesmata in the Casparian band region (Bonnett 1968). Hydrophobic interaction between the suberized cell wall of the Casparian band and membrane lipids or hydrophobic protein domains was, therefore, proposed (Bonnett 1968). This, however, does not fit with the occurrence of band plasmolysis in cells with Casparian bands that do not stain positive for lipid material using Sudan and berberine staining (Enstone and Peterson 1997), or with the ability of suberin lamella to detach the adhesion (Enstone and Peterson 1997; Haas and Carothers 1975). The involvement of transmembrane proteins acting as wall-to-membrane linkers, the existence of which was proposed in studies of Hechtian strands (Oparka 1994; Pont-Lezica et al. 1993), was also proposed, but the molecular background of the endodermal/exodermal plasmalemma adhesion has still not been resolved. Although CASPARIAN STRIP MEMBRANE DOMAIN PROTEINS (CASPs) are currently the most probable candidates, experimental evidence supporting their role as mediators of the adhesion is only indirect (Roppolo et al. 2011).

## 8.3 Endodermis, Hypodermis, and Exodermis: Basic Terminology

The terminology describing apoplastic barriers has developed over time. The name for the protective layer, "*Schutzscheide*," used by Caspary and other contemporary authors, was later replaced by "endodermis," a term originally reserved for the exodermal layer underlying velamen (Oudemans 1861). The term "exodermis" can be attributed to Vuillemin (1884), who defined it purely topologically as the outermost cortical layer under the epidermis. This subepidermal tissue, which is developmentally connected with the rest of the cortex, acts as a physiological boundary similar to epidermis. Pfitzer (1867) described it as "hypodermis," based on the term describing morphologically distinct layers under the epidermis of some

leaves (Kraus 1866). Similarly, Wiesner (1881; according to Kroemer 1903) thought the hypodermis equal to "external endodermis." The concept of the root hypodermis as a tissue cylinder of closely packed cells, with specific cell wall modifications providing functional support to the epidermis, was introduced by Meyer (1891). It should be mentioned that usage of the terms hypodermis and exodermis was far from consistent. The narrower use of exodermis in the sense of an endodermis-like hypodermis was most probably introduced by Rimbach (1893).

Within the hypodermis, there is a wide range of different tissue arrangements and their combinations with cell wall modifications. In this context, the excellent work of Kroemer (1903) should be mentioned, which outlines these arrangements and points out that some types of hypodermis (named "*Intercutis*") are composed not only of endodermis-like layers but might contain also rings of parenchyma, sclerenchyma, or cells containing Phi-thickenings. These parts are commonly considered to be non-exodermal parts of the hypodermis, whereas the exodermis is suberized tissue that develops Casparian bands (Enstone et al. 2003; Peterson and Perumalla 1990). The exodermal type of root hypodermis is the most common type among seed plants, which is why some authors, such as von Höhnel (1878), Wisselingh (1886), Haberlandt (1884), and Strasburger and Porter (1898), considered this layer to be general for vascular plants. Such an opinion was confirmed later by the studies of Perumalla et al. (1990) and Peterson and Perumalla (1990), indicating that a high percentage of seed plants develop an exodermal layer.

In spite of the similarity of endodermal and exodermal development, there are several features that seem to differentiate them. It was mentioned by Kroemer (1903) that exodermal cells in a primary state of development do not show the typical narrow points of Casparian bands (van Tieghem 1891, described by Kroemer 1903), but that developing Casparian bands and corrugated walls commonly cover radial cell walls almost completely. At this stage, suberization of the complete cell walls is commonly observed (Kroemer 1903). During the second stage, suberin lamellae are deposited over the internal surface of exodermal cells and frequently, but not necessarily, the compound middle lamella is lignified. It is often difficult to distinguish primary and secondary stages. Interestingly, their timing and the detectability of suberin lamellae and Casparian bands seems partially dependent on environmental conditions (Tylová et al. 2017). There is a high variation in secondary cell wall deposition patterns within the exodermis and a species-dependent arrangement of suberized and passage cells. Kroemer's systematic work should be acknowledged from the point of view of setting up the basic classification of exodermis types according to the presence of short passage cells, number of layers, and setup of differentiation according to root tip, which he documented for a wide set of species (Kroemer 1903). However, the last two features are strongly influenced by environmental conditions and should be viewed with reservation in the context of species-specific traits (Enstone and Peterson 1998; Enstone and Peterson 2005; Meyer et al. 2009; Soukup et al. 2002; Tylová et al. 2017).

## 8.4 Function of Apoplastic Barriers

The functional significance of the endodermis was expected from its very early discovery. Since then, there has been a long line of interesting discoveries. We have selected only a few and apologize for those not included. Caspary (1865) suggested the protective role of the endodermis, but without experimental evidence. Schwendener (1883) showed in *Iris* and *Convallaria* that a solution of iodine or tannins applied either from the cortical side or through the xylem vessels does not pass across the thick-walled cells of the *Schutzscheide*, but penetrates through the areas of thin-walled cells located opposite to the xylem poles in the subapical zone. He described these areas as "passages" and proposed their role in water transport from cortex to xylem vessels (Schwendener 1883). Schwendener (1883) considered that complete suberin lamellae and secondary cell wall in the fully differentiated state might act as a permeability barrier, but did not agree with the opinion that Casparian bands themselves might act as a barrier. However, he registered their resistance to sulfuric acid digestion and described the bands as cutinized. Schwendener considered Casparian bands to have purely mechanical significance and act as a tension resistive structure that counterbalanced the turgor difference between the central cylinder and cortex.

Hugo de Vries (1886; described by Kroemer 1903) named the endodermis *Kernscheede* (meaning core sheath, referring to the central cylinder). He conducted an interesting experiment, connecting a source of water under pressure to the base of the root and carefully removed the cortex not far away from the root tip. He did not observe any leakage of water and concluded that the endodermis and its Casparian bands act as a pressure barrier. Strasburger and Porter (1898) shared this opinion. Rufz de Lavison (1910) treated living roots with ferrous sulfate solution and histochemically detected sites of solute penetration. His experiments clearly indicated that it was endodermis with Casparian bands that acts as a barrier, which he described as a "living membrane" of the inner plant space. These results were later confirmed by Ziegenspeck (1921). In his textbook *Physiologische Pflanzenanatomie*, Haberlandt (1884) writes:

"It has already been mentioned above that the absorbing tissue (epidermis) of the roots with its root hairs is soon lost and slough off. The surface of the root is then covered by the outermost layer of cortex, the cell walls of which are corked and without intercellulars, and which now represents a complete secondary epidermis, so-called exodermis. If the corking of the walls takes place during the lifespan of the absorption tissue, short plasma-rich cells remain, which act as passage cells and transmit substances absorbed by the absorbent tissue to the cortical parenchyma (*Coleus*, *Lamium*, *Hedera*, and most monocots) among the long exodermis cells. Complete exodermis occurs however only with the air roots."

More complete descriptions of exodermis and its structural variability among species (Kroemer 1903) were soon followed by further experimental evidence.

In the early 1920s, experiments on root water suction force led Ursprung to propose that the endodermis functions simultaneously as a root pressure pump

(*Druckpumpe*) and as a check valve, allowing entry of water into the central cylinder and preventing its leakage back into the cortex (Ursprung and Blum 1921, 1923).

Priestley and coworkers published a series of papers presenting the endodermis as a semipermeable barrier to water and solutes that prevents the leakage of solutes from the stele and contributes to generation of root pressure (Priestley 1922; Scott and Priestley 1928; Wisselingh 1886). Priestley (1922) also emphasized the transport function of the primary endodermis with Casparian bands in an "absorptive region of root," in contrast to the transport resistance of the secondary endodermis with suberin lamellae in older root parts, where it closes the central cylinder and protects it from solution leakage and drying. In agreement, Rosene (1937) demonstrated longitudinal variation in water ingress rates along the root axis in onion and stressed the relationship between root length and position of the maximal ingress rate. Hayward and Spurr (1943) then showed that maximal water ingress in roots of *Zea mays* occurs in the zone with mature metaxylem vessels and primary unsuberized endodermis. They also showed that roots subjected to osmotic stress exhibit enhanced endodermis maturation and that the water uptake maximum shifts closer to the root tip in these roots (Hayward and Spurr 1943). These were pioneering studies, indicating that the endodermis is a crucial barrier with spatially variable permeability and is not a strictly impermeable boundary.

Later, the use of fluorescent dyes as probes to track apoplastic transport routes supported the idea that the endodermis and exodermis act as a barriers to apoplastic transport of compounds (Peterson and Edgington 1975), with significant spatial variation in permeability and the existence of leakage sites such as young immature root zones, sites of lateral root emergence, or wound sites (Aloni et al. 1998; Enstone and Peterson 1992; Faiyue et al. 2010; Moon et al. 1984; Peterson and Edgington 1975; Ranathunge et al. 2005a; Soukup et al. 2002, 2007). However, the barrier's tightness is significantly reinforced in response to adverse environmental conditions, which indicates the protective role of both layers. This is made clear in anatomical studies showing differentiation of the enhanced barrier. Accelerated maturation of Casparian bands and deposition of suberin lamellae occurs in the endodermis under stress conditions such as drought, salinity, and heavy metal toxicity (Enstone and Peterson 1998; Karahara et al. 2004; Lux et al. 2011; Redjala et al. 2011). In the exodermis, developmental plasticity is even stronger because this layer is functionally a "nonobligatory feature" with a protective role (Clarkson et al. 1987; Enstone and Peterson 1998; Kotula et al. 2009; Krishnamurthy et al. 2009; Meyer et al. 2009; Perumalla and Peterson 1986; Reinhardt and Rost 1995). In some species, the exodermis may only form in stress conditions (Reinhardt and Rost 1995; Zimmermann et al. 2000) or its differentiation constitutively shifts to precede endodermis maturation in species adapted to stress-bearing habitats such as wetlands (Soukup et al. 2002). Developmental plasticity thus seems to be the main factor setting the exodermis apart from endodermis, because the structural similarities in cell wall anatomy are quite obvious (Enstone et al. 2003; Geldner 2013;

Schreiber and Franke 2011; Van Fleet 1950) but species-specific structural variation of exodermis is far more extensive.

The barrier's permeability varies in relation to differentiation state, species, or environmental conditions (Schreiber and Franke 2011). Quantification of hydraulic parameters such as overall root hydraulic conductivity and its components (Bramley et al. 2007; Knipfer and Fricke 2010; Peterson et al. 1993; Ranathunge et al. 2003, 2005a, b; Steudle et al. 1993; Zimmermann and Steudle 1998), oxygen permeability (Armstrong and Armstrong 2001; Shiono et al. 2011; Soukup et al. 2007), or accumulation of adverse compounds within plant tissues from the surrounding environment (e.g., cadmium) (Redjala et al. 2011) have enabled a more precise understanding of the physiological role of these apoplastic barriers.

An interesting question is the different involvement of Casparian bands and suberin lamellae in setting up the barrier's transport properties. As already mentioned, the different transport properties of primary and secondary endodermis were proposed quite early (Kroemer 1903; Robards et al. 1973). Similarly for exodermis, Haberlandt (1884) suggested the function of short unsuberized passage cells (*Kurzzellen Intercutis*) in "transmission of material from absorbing tissues to living parenchyma of the cortex," in contrast to long suberized cells in the dimorphic exodermis (Strasburger and Porter 1898). Peterson and coworkers emphasized that passage cells with delayed suberin lamellae deposition are the only cells with accessible plasmalemma within the secondary endodermis. As such, passage cells may provide low resistance areas for movement of water and play a role in uptake of nutrients (e.g., calcium) that move preferentially by the apoplastic route and enter the symplast at the endodermal layer (Cholewa and Peterson 2004; Peterson and Enstone 1996).

The functional connection of nutrient uptake and barrier development is a topical area of current plant science (Andersen et al. 2015; Geldner 2013). Barberon et al. (2016) obtained experimental evidence, using fluorescence diacetate (FDA) as a tracer, that suberin lamellae indeed block direct uptake of compounds from the apoplast at the endodermal plasmalemma. However, suberin lamellae deposition excludes areas with plasmodesmata (Haas and Carothers 1975; Robards and Robb 1974; Waduwara et al. 2008) and does not hamper symplastic transport. Suberin lamella development thus makes the transition of endodermis from the primary state of "polarized epithelium," capable of transcellular transport of compounds, to the secondary state, a protective layer that only allows symplastic transport (Andersen et al. 2015; Barberon and Geldner 2014; Franke and Schreiber 2007; Geldner 2013). In agreement with the assumption of a functional symplastic route across suberized endodermis, substantial uptake of water was detected in suberized root areas (Sanderson 1983) as water uptake seems mostly restricted to the symplast (Knipfer and Fricke 2010; Ranathunge and Schreiber 2011). In agreement, the gradual maturation of endodermis/exodermis correlates with increasing frequency of aquaporins (channels facilitating water transport across membranes) within the endodermal/exodermal and epidermal plasmalemma (Hachez et al. 2006).

The role of the endodermis in root nutrient uptake and its selectivity is another essential question. It is textbook knowledge that the endodermis (with Casparian

bands and tight plasmalemma adhesion to the Casparian band cell wall region) is an obligatory feature for root uptake selectivity. However, this is not facile to prove it experimentally. Based on tracer studies, Schwendener (1883) proposed suberized endodermis as a barrier for transport of salts. Rufz de Lavison (1910) distinguished two groups of substances, those penetrating protoplasm that easily diffuse across the endodermis and those that do not penetrate the protoplasm and are stopped at the endodermis because of the presence of Casparian bands, as summarized by Scott and Priestley (1928). The importance of symplastic connection in root ion transport was emphasized by Arisz (1956) and Arnold (1952), who proposed that the endodermis has a function in solute accumulation (reviewed by van Fleet 1961). Bonnett (1968) emphasized the lack of studies combining active ion uptake with the fine structure of endodermal cells. Soon after, ion uptake was traced using radioactive analogs and showed higher phosphate but lower $Ca^{2+}$ uptake intensity in basal root regions compared with younger regions (Ferguson and Clarkson 1975). The decrease in $Ca^{2+}$ and $Mg^{2+}$ transport correlates with endodermal suberinization, whereas $K^+$ uptake is not affected (Ferguson and Clarkson 1975, 1976; Harrison-Murray and Clarkson 1973; Robards et al. 1973). Because $Ca^{2+}$ moves across the endodermis symplastically (Cholewa and Peterson 2004), suberinization seems to hamper the uptake of calcium into endodermal cells.

The set of available experimental tools for testing the role of the endodermis in nutrient uptake selectivity has been greatly extended by molecular biology methods, especially by isolation of mutant plants with disrupted Casparian bands. In mutants where defects in Casparian band lignification are compensated by enhanced endodermal suberinization (see Sect. 8.5), shoot elemental homeostasis is significantly affected. These mutants exhibit reduced levels of Mg, Ca, Mn, and Fe and increased levels of S, K, and Mo (Baxter et al. 2009; Hosmani et al. 2013). Surprisingly, only a mild nutrient uptake phenotype was found in different mutants with discontinuities in Casparian bands not compensated by oversuberinization (Pfister et al. 2014). These Casparian band defects are accompanied by lower root pressure and higher sensitivity to environmental conditions, but elemental homeostasis is only weakly affected. Potassium is the only essential element whose levels are significantly decreased (Pfister et al. 2014).

Studies focused instead on the impact of nutrient deficiency on endodermis/exodermis maturation gave inconsistent results. Although differentiation of both endodermis and exodermis is delayed under nitrate deficiency in roots of *Ricinus communis* (Schreiber et al. 2005), the wetland species *Carex gracilis* shows enhanced maturation of barriers in oligotrophic compared with eutrophic growth conditions (Končalová et al. 1993). *Zea mays* roots increase their suberinization under Mg deficiency (Pozuelo et al. 1984). Moreover, root hydraulic conductivity of *Zea mays* roots decreases under N deficiency but increases under K deficiency (Schraut et al. 2005), indicating nutrient-specific responses. Moreover, deficiency of K and S was recently shown to enhance endodermal suberinization via the abscisic acid signaling pathway, whereas Fe, Zn, and Mn deficiencies decrease suberinization via the ethylene signaling pathway (Barberon et al. 2016).

Nutrient-induced plasticity of endodermal differentiation may thus be an important adaptive ability of plant roots (Barberon 2017; Barberon and Geldner 2014; Barberon et al. 2016).

Considering the exodermis, the accessory apoplastic barrier of the outer cortex, its influence on root nutrient transport is even less clear. The maturation of exodermis significantly reduces the overall area of accessible plasmalemma within the root cortex (Kamula et al. 1994), thus contributing to the resistance to transport of radial water and solutes (Zimmermann and Steudle 1998) and probably impeding nutrient uptake. One of the few studies focusing on the role of the non-exodermal hypodermal layer in nutrient uptake by ion-selective microelectrodes showed that nitrate and ammonium uptake rate decline more sharply along the root axis in rice than in *Zea mays*, correlating with the presence of an additional sclerenchyma ring below the exodermis in rice roots (Colmer and Bloom 1998).

An interesting suggestion by von Guttenberg (1940) was the proposed role of the endodermis as a barrier to growth-regulating substances. This function of the endodermis was recognized recently (Dinneny 2014), for example, in auxin lateral redistribution during the phototropic response caused by relocation of PIN3 (PIN-FORMED 3) auxin efflux carrier on the endodermal plasma membrane (Ding et al. 2011) or in the role of the endodermis in abscisic acid distribution (Schraut et al. 2005).

## 8.5 Molecular Background of Endodermis Differentiation

Although discovery of the structural features of endodermal cell differentiation began more than a century ago, the molecular machinery involved in endodermis definition and establishment has only recently emerged. SCR (SCARECROW), SHR (SHORT-ROOT), and SCL23 (SCARECROW-LIKE 23), belonging to the GRAS (an acronym from GAI, RGA, and SCR) family of transcription factors (Pysh et al. 1999), were identified as central regulatory factors controlling root radial patterning and specification of the endodermal layer (Benfey et al. 1993; Di Laurenzio et al. 1996; Helariutta et al. 2000; Scheres et al. 1995). The *SCR* gene is expressed in cortex initials, and endodermal progenitor cells emerge via asymmetrical division of these initials (Di Laurenzio et al. 1996). In fact, knowledge of endodermal origin in cortical initials dates back to Caspary. *SHR* is expressed in stele and acts upstream of *SCR*, maintaining its expression in non-cell-autonomous mode (Helariutta et al. 2000; Levesque et al. 2006) and moving from the stele to an adjacent cell layer where it forms a SHR-SCR complex and specifies its endodermal fate (Nakajima et al. 2001). The distribution of SHR protein is restricted by dimerization to SCR and subsequent nuclear retention. This mechanism seems evolutionarily well conserved because a single-layered endodermis is common for vascular plants (Cui et al. 2007). Another identified co-player is SCL23 transcription factor, acting redundantly with SCR (Cui et al. 2014; Long et al. 2015a). SCR and SCL23 antagonistically regulate each other's expression. SCL23 exhibits

short-range mobility from ground tissue to stele and restricts outward movement of SHR; its cooperative activity with SCR is required to specify endodermal fate in the root meristem (Long et al. 2015a).

The action of SHR and SCR is further modulated by several members of the plant-specific C2H2 zinc finger protein family: JKD (JACKDAW), BIB (BALDIBIS), MGP (MAGPIE), and NUC (NUTCRACKER). These proteins physically interact with SCR and SHR and form nuclear complexes when coexpressed in the single cell (Long et al. 2015b; Welch et al. 2007). *JKD* is expressed early in the ground tissue in an SCR-independent manner, but later its maintenance becomes dependent on SCR and SHR (Welch et al. 2007). JDK and BIB (the closest homolog of JKD) act upstream of *SCR*, regulate *SCR* expression outside the stele by enhancing *SCR* promotor activity, constrain SHR-SCR complex movement to a single cell layer, and form a positive feedback loop that fortifies nuclear accumulation of SHR (Long et al. 2015b; Welch et al. 2007). Two other homologs, *MGP* and *NUC*, are downstream transcriptional targets of SCR and SHR that are both able to directly bind *MGP* and *NUC* promoters (Cui et al. 2007; Levesque et al. 2006; Welch et al. 2007). MGP and NUC promote SHR-dependent asymmetrical cell division of cortex/endodermis initials (Long et al. 2015b; Welch et al. 2007). Outside the root, SCR plays a corresponding role in specification of leaf bundle sheaths and starch sheaths of the stem. Both of these structures are analogous to the endodermis and might in some cases exhibit its typical structural features. The *zmscr* mutant of *Zea mays* has proliferated bundle sheath cells and several abnormalities in leaf anatomy (Slewinski et al. 2012). Incorrect definition of starch sheaths in allelic *scr* and *shr* mutants impairs shoot gravitropism (Fukaki et al. 1996, 1998).

The onset of endodermal differentiation is directed by MYB36 (MYB DOMAIN PROTEIN 36) transcription factor (Kamiya et al. 2015; Liberman et al. 2015). The expression of *MYB36* is directly activated by SCR and subsequently regulates the expression of genes involved in endodermal cell wall modifications such as Casparian band formation (Liberman et al. 2015). The *myb36 Arabidopsis* mutant shows delayed and defective endodermis differentiation, irregular endodermal lignification instead of well-established Casparian bands, and disrupted endodermal barrier function (Kamiya et al. 2015; Liberman et al. 2015). Ectopic *MYB36* expression in cortical cells triggers activation of the Casparian band building machinery (Kamiya et al. 2015).

Casparian band formation is the first and obligatory phase of endodermal cell wall modification (Enstone et al. 2003; Geldner 2013). Formation of the band starts with delineation of the median plasmalemma domain (CSD; Casparian strip membrane domain) by specific membrane proteins, CASPs (Roppolo et al. 2011). These proteins are transported to the plasmalemma in a nonlocalized manner, but later accumulate exclusively in the CSD, mark it, and remain there as a stabilizing transmembrane scaffold required for precise localization of the lignin deposition machinery (Lee et al. 2013; Roppolo et al. 2011; Roppolo and Geldner 2012). The CSD separates outer and inner polar domains of the endodermal plasmalemma and prevents lateral membrane diffusion between these domains; its establishment

clearly precedes lignification of the Casparian band (Alassimone et al. 2010; Roppolo et al. 2011). In *Arabidopsis*, the CASP family contains five genes encoding four transmembrane-spanning proteins with a conserved nine amino acid signature within the first extracellular loop (Roppolo et al. 2014). Related CASP-like proteins (CASPL) without such a signature are expressed in other plant tissues, where they can form similar membrane fences/scaffolds, but their individual functions have not yet been experimentally elucidated (Roppolo et al. 2014). CASP genes display some redundancy; only the *casp1casp3* double mutant (not single *casp1* or *casp3* mutants) showed visible defects in endodermal lignification, surprisingly without loss of function as an apoplastic barrier and with a very mild growth phenotype (Roppolo et al. 2011).

The precise localization of CASPs in the CSD is a tightly regulated process that has recently been unraveled. Among others, EXO70A1, a subunits of secretory complex exocyst, seems to be involved because *exo70a1*/*lotr2* (*lord of the ring 2*) plants show dramatic delocalization of CASPs (Kalmbach et al. 2017). Altered localization of CASPs, disrupted Casparian bands, and ectopic endodermal suberinization are also results of *lotr1* (*lord of the ring 1*) mutation. Although *LOTR1* expression is not endodermal specific and LOTR1 function remains unclear, its involvement in cell wall modification influencing lateral membrane diffusion of CASPs has been hypothesized (Li et al. 2017).

Another player involved in Casparian band positioning and establishment is the CIF-SGN3-SGN1 signaling module (Doblas et al. 2017). SCHENGEN3 (SGN3)/GASSHO1 is a leucine-rich receptor-like kinase (LRR-RLK) of subfamily XI (Tsuwamoto et al. 2008) located in the plasmalemma around the forming CSD and is required for proper CASP protein localization into the CSD (Pfister et al. 2014). SGN3 is activated by CIF1,2 (CASPARIAN STRIP INTEGRITY FACTOR 1,2) peptide ligands (Doblas et al. 2017; Nakayama et al. 2017). Stele-expressed CIF peptides, sulfated by TPST/SGN2 (TYROSYLPROTEIN SULFOTRANSFERASE/SHENGEN 2), move toward the endodermis to active SGN3 (Doblas et al. 2017). The CIF-SGN3 signal is further transduced by SGN1 (SCHENGEN1) receptor-like cytoplasmic kinase (RLCK) of subfamily VII. SGN1 is localized in a polarized manner to the cortex-facing domain of the endodermal plasmalemma (Mohl 1847). The CIF-SGN3-SGN1 signaling module might present an interesting mechanism for control of endodermal barrier tightness by the movement of stele-derived apoplastic ligands that activate the Casparian band formation machinery (Doblas et al. 2017). It might also modulate Casparian band establishment in response to environmental nutritional clues because excess iron enhances CIF expression, leading to reinforcement of the barrier (Nakayama et al. 2017). All *sgn1-3* and *cif1cif2* mutants display similar endodermal defects. CASP protein plasmalemma islands do not fully fuse into the CSD, which leads to formation of interrupted Casparian bands (Nakayama et al. 2017; Pfister et al. 2014). Surprisingly, the *sgn3* mutant displays only a mild nutrient uptake phenotype, although it is sensitive to abiotic stress. Potassium is the only nutrient that accumulates in significantly lower amounts in *sgn3* compared with wild-type plants (Pfister et al. 2014). The *cif1cif2* double mutants are hypersensitive to excess iron (Nakayama et al. 2017).

The molecular machinery for modification of the Casparian band cell wall was unveiled recently. In accord with previous conclusions, lignin or lignin-like material was confirmed as a key part of the Casparian bands in *Arabidopsis* (Naseer et al. 2012). Almost all the suberin biosynthetic genes are activated after the onset of Casparian bands. Moreover, selective manipulation of suberin or lignin biosynthesis in *Arabidopsis* roots confirmed the importance of aromatic monomers (lignin) but not aliphatic constituents of suberin in building functional Casparian bands under the conditions used (Naseer et al. 2012). Even in *Arabidopsis*, the synthetic enzyme ASFT (ALIPHATIC SUBERIN FERULOYL TRANSFERASE) is transcriptionally activated very early during Casparian band development (Naseer et al. 2012). ASFT is required for the linkage of ferulate monomers into fatty acids (Molina et al. 2009) and thus might integrate some ferulic compounds into emerging Casparian bands (Naseer et al. 2012). In our opinion, the declared absence of aliphatic monomers/domain of suberin from Casparian bands is a matter for question, at least in other plant species. Analytical chemistry indicated substantial amounts of aliphatic suberin in *Zea mays* endodermis in the primary stage of differentiation (Casparian bands only), which differed in composition from the suberin deposited during the secondary stage as typical suberin lamellae (Zeier et al. 1999). Similarly, the endodermal cell walls of *Clivia minimata*, which do not develop after the primary stage of endodermal development (do not form any suberin lamellae), contained high amounts of aromatic lignin, which had a quantitative monomeric composition different from that of xylem vessel lignin (Schreiber 1996; Zeier and Schreiber 1997). Aliphatic suberin monomers originating from the Casparian band were clearly detected in this case.

Deposition of lignin polymer into the cell wall above the CSD is driven by a specific cell wall peroxidase that converts monolignols into radicals for subsequent oxidative coupling; NADPH oxidase provides the necessary $H_2O_2$. In *Arabidopsis* endodermis, a specific peroxidase PER64 and the NADPH oxidase RBOHF (RESPIRATORY BURTS OXIDASE HOMOLOG F) are involved, both specifically localized in emerging Casparian bands (Lee et al. 2013). PER64 requires CASP1 protein for proper apoplastic localization above the CSD but not for secretion itself (Lee et al. 2013). Lignin polymerization within the Casparian band also requires dirigent domain-containing protein ESB1 (ENHANCED SUBERIN 1). ESB1 is an endodermis-specific protein localized in emerging Casparian bands in a CASP-dependent manner (Hosmani et al. 2013) that is thought to guide the stereochemistry of emerging lignin polymer by acting as a template for proper monolignol orientation (Davin and Lewis 2000, 2005). Loss of ESB1 function results in defects in CASP1 localization and Casparian band formation that are compensated with ectopic root suberinization (Hosmani et al. 2013). Monolignol precursors of lignin–hydroxycinnamoyl alcohols are transported into the cell wall prior to polymerization (Boerjan et al. 2003; Voxeur et al. 2015). Monolignols are transported across the plasmalemma or tonoplast by transporters containing ATP-binding cassettes (Miao and Liu 2010). In endodermis, the only transporter characterized so far is the *p*-coumaryl alcohol exporter AtABCG29, located outside the CSD in endodermal plasmalemma (Alejandro et al. 2012;

Roppolo and Geldner 2012). The broader function of this transporter is obvious, as it is also present in *Arabidopsis* vascular tissue. The *abcg29* mutant shows reduced levels of *p*-hydroxyphenyl lignin subunits and also of quaiacol and syringyl subunits and some flavonoids and glucosinolates (Alejandro et al. 2012).

Formation of suberin lamellae is the second (not obligatory) phase of endodermal cell differentiation that further reinforces the apoplastic barrier function of the layer in a manner that does not duplicate the function of Casparian bands (Andersen et al. 2015; Enstone et al. 2003; Geldner 2013). Suberin is a lipid-phenolic biopolymer, and its deposition starts with synthesis of aliphatic, phenolic, and glycerol monomers that are later transporter across the plasmalemma. The suberin biosynthetic pathway has been the subject of several reviews (Bernards 2002; Nawrath et al. 2013; Ranathunge et al. 2011; Vishwanath et al. 2015). Among the genes specifically expressed in root endodermal cells, *CYP86A1*/*HORST* encodes cytochrome P450 fatty acid ω-hydroxylase localized in the endoplasmic reticulum. Mutant *cyp86a1*/*horst* plants showed significant reduction in $C_{16}$–$C_{20}$ ω-hydroxyacids, resulting in 60% reduction in the amount of total aliphatic suberin in roots (Höfer et al. 2008). Related *CYP86B1*/*RALPH* has a similar endodermal expression pattern and protein localization, but the enzyme is involved in hydroxylation of $C_{22}$–$C_{24}$ fatty acids (Compagnon et al. 2009). ASFT (ALIPHATIC SUBERIN FERULOYL TRANSFERASE) is an acyltransferase with endodermal (but also periderm and seed coat) localization and is essential for ferulate incorporation into suberin (Molina et al. 2009). Moreover, some other ABCG transporters (ABCG2, ABCG 6, and ABCG20) were shown to mediate transport of suberin monomers in endodermis (Yadav et al. 2014). The *abcg2,6,20* triple *Arabidopsis* mutant shows altered aliphatic suberin composition, increased endodermis permeability of older root parts, but fully functional Casparian bands (Yadav et al. 2014). Similarly, the *rcn1*/*osabcg5* (*reduces culm number1*) mutant of *Oryza sativa* shows decreased levels of root aliphatic suberin monomers and more permeable hypodermal layers. *RCN1*/*OsABCG5* expression increases in rice hypodermis and to certain extent also in endodermis under stagnant deoxygenated conditions (Shiono et al. 2014).

The deposition of a cellulosic "tertiary wall" is the third (not obligatory) stage of endodermal/exodermal cell wall modification. It most probably functions as mechanical support and is very common in monocots (Enstone et al. 2003). Molecular mechanisms driving this typically asymmetric secondary cell wall deposition are still unclear. Master regulators of secondary cell wall deposition, such as NAC transcriptional factors NST1-3 (NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1-3) and SND1 (SECONDARY WALL-ASSOCIATED NAD DOMAIN PROTEIN 1) or MYB transcriptional factors MYB46, MYB83, MYB58, and MYB63, are involved in cell wall thickening of different plant tissues (fibers, vessels, anther endothecium or siliques) but an endodermal-specific role has not been indicated for any of them (Mitsuda et al. 2005, 2007; Zhong et al. 2007, 2011; Zhou et al. 2009). In some grasses (e.g., *Sorghum*), silica aggregates in the inner tangential wall of endodermal cells may further reinforce the mechanical properties of the layer (Kumar et al. 2017; Soukup et al. 2014).

Other important features of endodermal/exodermal cells are tight membrane adhesion to the cell wall within the Casparian band domain and lateral polarity of the endodermal/exodermal plasmalemma. The tight adhesion support barrier function of the layer can be visualized as band plasmolysis (see Sect. 8.2) and persists until suberin lamella is deposited (Enstone and Peterson 1997; Karahara et al. 2004). The molecular background of adhesion is unclear. CASP proteins might participate, although experimental evidence is indirect (Roppolo et al. 2011). Candidate SGN3 receptor-like kinase is clearly not involved because membrane adhesion persists in *sng3* plants (Pfister et al. 2014).

The lateral polarity (inner and outer domain) of the endodermal/exodermal plasmalemma is a feature related to the transport function of the layer. There are polarized influx and efflux transporters in the outer and inner membrane regions of the endodermal/exodermal plasmalemma separated by CSD, including boron transporters (BOR1, NIP5;1), silicon transporters (Lsi1, Lsi2), and an auxin efflux carrier PIN3 (Ding et al. 2011; Ma et al. 2006, 2007; Ma and Yamaji 2015; Takano et al. 2006, 2008, 2010). From this point of view, the barrier layer resembles animal epithelia with tight junctions (Alassimone et al. 2010, 2012; Barberon and Geldner 2014). Lateral polarity is, however, not an exclusive feature of cells bearing a Casparian band. Polarized localization of nutrient transporters is also documented for rhizodermis (Barberon et al. 2014; Barberon and Geldner 2014; Miwa et al. 2007), root apex (Takano et al. 2010), and middle cortex (Mitani et al. 2009b). Positioning of transporters is a dynamic feature that is established in response to nutritional/environmental clues (Barberon and Geldner 2014; Ding et al. 2011) as well as species-specific nutritional demands (Ma and Yamaji 2015; Mitani et al. 2009a).

## 8.6 Conclusions

The data summarized here clearly show that apoplastic barriers with their functional and structural aspects, development, and ecophysiological significance for plant survival are a very important current research theme with a long tradition and high potential for the future. We hope that this simplified review will help readers in orientation within the topic.

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# Chapter 9
# Evolving Views on Plastid Pleomorphy


**Kathleen Delfosse, Michael R. Wozny, Cole Anderson, Kiah A. Barton, and Jaideep Mathur**

**Abstract** The presence of organelles named "plastids," recognized most commonly for conferring autotrophy through photosynthesis, is considered a distinguishing feature of the Viridiplantae. Plastid identity is not fixed, because function and contents differ according to the position and energy requirements of the cell in which the plastid is found. Similarly, it is difficult to ascribe a typical form to a plastid in a living plant cell because their appearance varies considerably between tissues and plastid types, as well as in response to environmental factors. Although diverse aspects of plastids, especially chloroplasts, have been studied assiduously over the past two and a half centuries, the mechanisms underlying their pleomorphy have remained enigmatic. This review dissects the key features of plastids that may contribute to their shape and discusses recent observations and ideas surrounding plastid pleomorphy.

## 9.1 The "Plastid" Organelle

Although ancient botanical texts dating back several thousands of years recognize the greenness and other properties of plants (Raghavendra et al. 2003), modern literature credits Leeuwenhoek with the first documented observations of green organelles within algae and higher plants (1674, recorded in Dobell 1932). The term "plastid" was introduced into biology by Haeckel (1866; Lankester 1876) to encompass what he defined as the first-order structures or "form units" of an organism. These structures included nucleated cells and anucleate structures called cytods, which presumably represented subcellular organelles. Schimper (1882) adopted the term to apply exclusively to the subcompartments within plant cells, today recognized as plastids. The variability of form and function shown by these

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organelles appears to be the underlying basis for this choice, as the word ”plastid” is traceable to the Greek *plastikos*, meaning that which can be molded. Schimper's realization that several organelles known individually as *Chlorophyllkörper* (chloroplasts), *Leukoplastiden* (leucoplast), *Stärkebildner* (amyloplasts), and *Farbkörper* (chromoplasts) could all be traced to colorless pro-plastids emphasized the pleomorphy of a single organelle and allowed them to be grouped under a single term (Schimper 1882).

Schimper (1883) was also the first to suggest that chloroplasts resembled cyanobacteria and could be symbionts within plant cells. This notion was further developed by Mereschkowsky (1905; annotated by Martin and Kowallik 1999) and brought into mainline biological theory by Lynn Margulis (Sagan 1967). Endosymbiosis of photosynthetic cyanobacteria leading to the stable acquisition of plastids is postulated to have occurred at least once in Earth's evolution, giving rise to the double membrane-enclosed primary plastids. These plasmids include the peptidoglycan-walled muroplasts in glaucocystophytic algae, the rhodoplasts of red algae, and the green lineage plastids found in the Viridiplantae. Plastids with three or four membranes occur in some organisms and are attributed to secondary or tertiary endosymbiotic events (Larkum et al. 2007).

The differentiated forms of primary plastids in the Viridiplantae are still known today by the pigmentation-based nomenclature introduced in the nineteenth century. Under this nomenclature, chloroplasts are characterized by the predominance of chlorophyll, chromoplasts by the predominance of other pigments, and leucoplasts by the absence of pigmentation (Schimper 1882; Haberlandt 1901; Gunning et al. 2007). The increased complexity of tissue differentiation initiated in the streptophyta and developed further in the true embryophytes has led to major differences in the predominant inclusions of plastid populations. This has led to the naming several leucoplast subgroups as amyloplasts, elaioplasts, and proteinoplasts, which are plastids that predominantly store starch, lipids, and protein, respectively. Additional groups of plastids have been identified based on the developmental stage of the plant. Aerial tissues in seedlings grown without light contain protochlorophyllide-rich plastids called etioplasts, whereas senescent aerial tissues contain gerontoplasts that aid in the breakdown and remobilization of photosynthetic components and internal membranes (Wise 2007; Pyke 2009). The predominant plastid population changes according to the tissue, as well as developmental and environmental requirements, highlighting another characteristic of plastids – their interconvertibility.

Pro-plastids in vegetative aerial meristems generally differentiate into chloroplasts; however, under low light or dark conditions they become etioplasts. These in turn develop into chloroplasts if triggered by light (Gunning 1965; Kowalewska et al. 2016). Similarly, chloroplasts can become chromoplasts in ripening fruits, and either chromoplasts or leucoplasts in petals (Pyke 2009; Egea et al. 2011). Carotenoid-containing chromoplasts in the roots of carrot (Fuentes et al. 2012; Rodriguez-Concepcion and Stange 2013) and colorless leucoplasts in the roots of other species can undergo conversion into chloroplasts upon exposure to light (Usami et al. 2004).

There also exists variability in function within a plastid subgroup, as demonstrated by amyloplasts, which can be derived from plastids in both roots and aerial tissues. Amyloplasts can function in gravitropism or storage depending on cell type (Salema and Badenhuizen 1967; Hashiguchi et al. 2013; Borucki et al. 2015; Matsushima et al. 2016). Although the identity and function of a plastid are clearly not fixed characteristics, several features are universal to all plastid types.

## 9.2 Basic Features of Plastids in Viridiplantae

The plastids of the Viridiplantae are delineated by an envelope consisting of outer and inner membranes. Most of the plastid volume is filled with a semi-aqueous, soluble-protein-rich matrix called the stroma (Weier 1938; Gunning et al. 2007; Wise 2007). The outer envelope membrane has historically been considered freely permeable to most soluble metabolites, with the inner envelope membrane serving as the major physical barrier to diffusion between the cytosol and the stroma (Block et al. 2007). However, as evidenced by the presence of numerous selective transporters in the outer membrane, metabolite trafficking across both envelope membranes is highly regulated (Weber and Linka 2011). The high selectivity is demonstrated by proteins such as outer envelope protein 40 (OEP40), which is permeable to glucose, glucose-1-phosphate, and glucose-6-phosphate but not to maltose (Harsman et al. 2016). Protein import is also regulated at both membranes, through the complexes making up the translocons of the outer envelope membrane (TOC; Lin and Jarvis 2015) and the inner envelope membrane (TIC; Jarvis 2008; Kovács-Bogdán et al. 2010). Lipids are known to move between the envelope membranes with the aid of transporters that span the outer and inner envelope membranes, such as the trigalactosyldiacylglycerol (TGD) proteins that form the TGD1/2/3/4 complex (Kurlock et al. 2014).

As a strong indication of their postulated prokaryotic origin, plastids have circular chromosomes that are found in complex with proteins and RNAs as nucleoid structures. They also have their own ribosomes, which contain many proteins that are orthologous to prokaryotic ribosomal proteins (Yamaguchi and Subramanian 2000). Many plastids also exhibit some internal membrane structure, with thylakoids that are stacked into grana in most chloroplasts but show less defined structure in other plastid types (Pyke 2007). Common internal features of plastids also include inclusions such as lipid monolayer-bound particles called plastoglobuli (Austin et al. 2006) and starch grains composed of glucose polymers (Smith et al. 1997; Zeeman et al. 2010). The degree of internal membrane structure and the accumulation of inclusions vary depending on plastid type and tissue.

Another commonality between plastids is their tendency to exhibit pleomorphy, a continuous change in shape. Transmission electron microscopy snapshots show a general spherical to oval outline for the organelle. However, light microscopy of living specimens has provided an exciting view of the dynamic plant cell and laid down the foundations for our present ideas on plastid pleomorphy.

## 9.3 Observations of Plastid Pleomorphy

Some of the first observations of dynamics within the plant cell are traced to Corti (1774; reviewed in Allen and Allen 1978), who reported the bulk streaming of cytoplasmic components, including plastids. Clear observations that highlighted the dynamic shape of chloroplasts followed nearly a century later (Sachs 1859; Micheli 1867). Although the changes observed were initially attributed to exposure to light, subsequent investigations by Stahl (1880), Schimper (1885), and Senn (1908) suggested links to other external conditions (Zurzycki 1964). Observations on isolated chloroplasts established the presence of a clear plastid envelope (Mudrack 1956), and investigations using cine-photomicrographic techniques revealed fresh details of subcellular motility and organelle behavior (Wildman et al. 1962; Green 1964). The chloroplast envelope was found to be very dynamic and was likened to a mobile jacket, constantly changing its shape while the chlorophyll-containing grana inside remained motionless (Spencer and Wildman 1962). Sporadically, long colorless protuberances were seen to extend from the chloroplasts into the surrounding cytoplasm (Esau 1944; Wildman et al. 1962). Leucoplasts lack the more consistent shape of the chloroplast and, in addition to forming protuberances, can undergo whole-plastid changes in shape (Esau 1944).

The presence and dynamic nature of plastid extensions was firmly established through observation of plastids labeled with a stroma-targeted green fluorescent protein (GFP; Köhler et al. 1997). The long, thin stroma-filled tubules that extend from a plastid body were named "stromules" (Köhler and Hanson 2000). Observations on stromules in a wide variety of plants and tissues have established them as a basic feature of plastids. They are believed to increase the interactive surface between a plastid and the surrounding cytoplasm (Holzinger et al. 2007a, b; Schattat et al. 2012a). It has also been suggested that stromules may serve as bridges between plastids to allow exchange of metabolites (Köhler et al. 1997; Tirlapur et al. 1999; Gray et al. 2001). However, long-term observations have failed to reveal any evidence of fusion between independent plastids, opposing a proposed role in interplastid exchange (Schattat et al. 2012b).

Investigations on stromules have opened several avenues of exploration into the cause and mechanism of their formation (Natesan et al. 2005; Gunning 2005; Shaw and Gray 2011; Schattat et al. 2014; Delfosse et al. 2016), but it must be remembered that the extension and retraction of long stromules is just one manifestation of the general phenomenon of plastid pleomorphy. A change in plastid shape is already apparent when tiny protrusions of the envelope give an undulating outline to plastids in living cells. Tightly crowded mesophyll chloroplasts are often reported to have numerous protrusions, but few stromules (Holzinger et al. 2007a, b; Buchner et al. 2007; Hanson and Sattarzadeh 2008; Moser et al. 2015). The term "chloroplast protrusion" (CP) is used to describe short, wide protuberances that are sometimes considered a phenomenon distinct from stromule formation (Holzinger et al. 2007a, b; Lütz 2010). However, stromules are initiated as protrusions of the envelope indistinguishable from CPs and return to a similar state as they retract into

the plastid body (Delfosse et al. 2016); whether there is a functional or structural distinction between the structures requires further investigation. Other protuberances of various lengths and shapes have also been described in the literature, with many sources aptly referring to the amoeboid nature of the plastid (Newcomb 1967; Valanne and Valanne 1972; Bonzi and Fabbri 1975; Bourett et al. 1999; Wise 2007). These observations all attest to the pleomorphic behavior of plastids. A number of ideas have been formulated to account for this phenomenon; however, no universal mechanism or explanation for plastid pleomorphy has been provided to date and many factors, both internal and external to the plastid, may play a role.

## 9.4 Effects of Intraplastidial Membranes on Overall Plastid Morphology

The stroma and the envelope membranes of plastids are flexible enough to accommodate internal membranes, such as pro-lamellar bodies and thylakoid membranes, and the structure of these membranes often correlates with plastid behavior. Mesophyll chloroplasts generally have well-defined internal membrane structures, with thylakoids stacked into rigid grana (Vothknecht and Westhoff 2001). These chloroplasts are consistently described as discoid or lens-shaped (Esau 1944; Mego and Jagendorf 1961; Thomson and Whatley 1980), and this shape has been reported to develop alongside development of the grana (Vothknecht and Westhoff 2001). In $C_4$ plants, a distinction in shape is seen between the mesophyll and bundle sheath chloroplasts, which is attributed to differences in thylakoid stacking (Munekage 2016). Furthermore, based on the presence of chlorophyll and grana, the plastids in epidermal pavement, guard, and mesophyll cells in light-grown *Arabidopsis* plants can all be classified as chloroplasts (Pyke 2009; Barton et al. 2016). However, the chloroplasts of these different types of leaf cells display differences in morphology alongside differences in internal membrane structure. Chloroplasts in epidermal cells have fewer, smaller grana than mesophyll chloroplasts (Dupree et al. 1991; Barton et al. 2016) and correspondingly tend to be less rounded and more prone to the extension of stromules (Kwok and Hanson 2004b). In contrast to the structure of chloroplasts, the internal membranes in leucoplasts are generally accepted to be minimal and to lack a defined structure. Correspondingly, the shape of these plastids is undefined, with time-lapse imaging showing them to be very flexible and dynamic in shape (Schattat et al. 2012a, 2014). A role for internal membranes in shaping the plastid is suggested by these observations.

## 9.5 Carbohydrate Metabolism and Plastid Pleomorphy

Just as plastid morphology is plastic, so too are the metabolic roles that plastids serve. The terms ”source” and “sink” have been used to describe particular plants tissues based on their tendency to predominantly export or import energy-rich

metabolites. Photosynthetic tissues such as leaves synthesize usable fixed-carbon metabolites and are thus considered carbon-source tissues. However, carbohydrates must be imported to the developing shoot and root tissues to sustain cell metabolism when photosynthetic chloroplasts are not present or not capable of meeting cellular demands. For example, newly emergent expanding leaves consume most of the photo-assimilates that they are able to produce and rely upon the import of carbohydrates for growth until they reach 30–60% of their final surface area. At this point, the leaves gradually shift to exporting the majority of their photo-assimilates, transitioning from a sink to a source (Turgeon 1989).

In addition to the cell's developmental stage, the photosynthetic capability and carbon reserves of its resident plastids are key criteria for the establishment of sink or source status (Roitsch 1999; Osorio et al. 2014). During photosynthesis, chloroplasts produce photosynthates in the form of triose phosphates (TPs), which are primarily exported to the cytosol via the triose-phosphate/phosphate translocator (TPT) (Preiss 1984; Flügge and Heldt 1991). TPs in the cytosol are then used for sucrose synthesis and distributed throughout the plant. A portion of the TPs are retained in the chloroplast and directed toward transitory starch synthesis. Thus, during the light period, active chloroplasts are simultaneously carbon sources and sinks. During the night, chloroplasts cannot fulfil source requirements through photosynthesis. They do so instead by remobilizing transitory starch through its degradation into maltose, which is subsequently exported to the cytosol (Weise et al. 2004, 2005) via the maltose transporter MEX1 (Niittylä et al. 2004). Starch degradation and maltose export are regulated both by an endogenous circadian control mechanism and by light (Lu et al. 2005). Amyloplasts, such as those of potato root or cereal endosperm tissue, can also act as both a sink and a source. During their development, amyloplasts accumulate large amounts of starch through import of sugars; however, they can function as carbon sources through starch degradation and energy remobilization when required (Kelly and Latzko 2006).

There is an apparent connection between plastid pleomorphy and starch accumulation within a plastid. Starch grains have a well-defined rigid structure, and starch-filled amyloplasts are consistently described as swollen, with a spherical or slightly elliptic shape (Salema and Badenhuizen 1967; Thomson and Whatley 1980; Sagisaka 2008). Similar plastid morphology is seen in the chloroplasts of starch-accumulating mutants (Matsushima et al. 2016). Furthermore, there is often a difference in starch content and thylakoid stacking between the chloroplasts of mesophyll and bundle sheath cells (Munekage 2016). In rice, chloroplasts within bundle sheath cells are known to accumulate more starch and appear more oblong in shape than mesophyll chloroplasts (Sage and Sage 2009; Miyake 2016).

It therefore appears that plastid morphology is influenced by metabolic state through starch accumulation and degradation. For amyloplasts, which are generally responsible for long-term carbon storage, changes are on a developmental timescale. In contrast, chloroplasts show much more rapid accumulation and degradation, regulated by light and the circadian cycle.

Interestingly, the formation of protrusions (Buchner et al. 2007; Moser et al. 2015) and stromules (Schattat et al. 2012a; Brunkard et al. 2015) is known to be

light responsive. This phenomenon is postulated to be caused by either a change in redox status (Brunkard et al. 2015) or by the cytosolic accumulation of sucrose during photosynthesis (Schattat et al. 2012a). The relationship between stromule frequency and cytosolic carbohydrate levels is strengthened by the rapid increase in the proportion of epidermal chloroplasts with stromules in *Nicotiana benthamiana* and *Arabidopsis thaliana* in response to exogenous sugar treatment (Schattat and Klösgen 2011; Schattat et al. 2012a).

Given the correlation between starch-promoting conditions and stromule extension, it would be interesting to investigate whether starch accumulation or degradation affects plastid membrane extensions. Small starch granules have been observed at the tips of protrusions and stromules as well as in bulges within stromules, suggesting that starch can change stromule morphology and influence their extension (Langeveld et al. 2000). Just as a large starch-filled body can constrain the shape and movement of a plastid, smaller starch granules could exert an outward pressure on the envelope as they grow, promoting the formation of extensions. Alternatively, small starch grains within the plastid could become trapped through physical interactions with other cellular components. If the plastid body moved in relation to these trapped starch granules, the plastid membranes could be stretched into a thin tubule. Such an occurrence could explain the appearance of small starch granule-like structures that are sometimes observed and appear to be separate from larger starch granules and the plastid body (Wang and Liu 2013). Other plastid inclusions, including plastoglobuli and crystalline structures, could theoretically play a similar role to starch in modifying plastid shape. Whether the accumulation of starch or other inclusions affects plastid mobility and stromule formation remains an open question. Altering a plastid's source and sink activities by manipulating the accumulation or mobilization of photosynthates could prove to be an exciting avenue of investigation into plastid morphological responses. Sugars derived from photosynthesis in source tissues can also feed into other metabolic pathways, including lipid synthesis pathways that contribute to the formation and maintenance of the plastid envelope and other cellular membranes.

## 9.6 Possible Role for Lipids in Determining Plastid Shape

Lipid synthesis in plant cells is dependent upon the synthesis of fatty acids (FAs) within the plastid stroma and the subsequent incorporation of FAs into plastid-synthesized lipids or their export from the plastid for assembly into ER-synthesized lipids (Koo et al. 2004). Additionally, plastid-synthesized lipids may be exported for desaturation in the ER, by ER-localized desaturases, and subsequently returned to plastid membranes (Bates et al. 2007; Tjellström et al. 2012). The lipid profile of a membrane plays a key role in its behavior, and different lipid species promote different forms of membrane curvature (Jouhet 2013). A change in the lipid profile of a membrane could therefore theoretically influence the morphology of the organelle it defines (Jarsch et al. 2016).

External factors can effect rapid changes in membrane properties. For example, a decrease in temperature promotes membrane rigidity, and the cell responds through the desaturation of lipids. This increases the fluidity of the membrane and maintains homeoviscosity (Sinensky 1974). Conversely, increased temperature results in a more fluid membrane prior to the cell increasing saturation to compensate. Both desaturation in response to cold stress and saturation in response to heat have been demonstrated to occur in plants (Welti et al. 2002; Tasseva et al. 2004; Larkindale and Huang 2004). In addition to the changes in lipid saturation, in some bacteria the composition of membranes in terms of lipid species is regulated to maintain the fluidity of cell membranes (Lindblom et al. 1986, 2002), and changes to the lipid species in plant cells in response to temperature have been observed (Szymanski et al. 2014). The rapid formation of plastid protrusions has been reported in response to increasing temperature (Holzinger et al. 2007a; Buchner et al. 2007). Given the changes in fluidity that heat causes in a membrane, it is possible that protrusions result from changes in the fluidity of the plastid envelope membranes. This suggests that plastid shape is directly affected by the lipid species within the plastid envelope, the saturation of those lipids, and environmental temperature. Furthermore, different lipid species promote different forms of curvature depending on their head group, chain length, and chain saturation. Certain lipids promote the formation of tubules in vitro, and highly curved membranes such as ER tubules or thylakoid edges are thought to be promoted by the presence of certain lipid shapes (Jouhet 2013). The idea that regions of similar lipids in a plastid membrane could promote changes in plastid behavior or the tubulation of its membranes is intriguing.

The production of FAs and lipids that can influence membrane composition is a complex process that is dependent on the coordination of plastid and ER localized pathways. Lipid trafficking between plastids and other organelles probably does not result from vesicular trafficking because plastids are regarded as being largely disconnected from the endomembrane transport system (Villarejo et al. 2005; Jarvis and López-Juez 2013). The process of exchange is instead thought to occur through membrane contact sites (MCSs; Block and Jouhet 2015). MCSs could be partly responsible for any lipid-induced effect on plastid pleomorphy as possible conduits for lipid transfer to and from the plastid (Wang and Benning 2012). Speculation that MCSs are important drivers in the formation of stromules has led to the suggestion that stromules are affected by conditions that increase lipid synthesis and trafficking between plastids and other organelles (Block and Jouhet 2015).

An increase in lipid exchange between plastids and extraplastidial membranes has been well characterized during times of nutrient stress, including phosphate (Essigmann et al. 1998; Andersson et al. 2003, 2005; Jouhet et al. 2004; Nakamura et al. 2005; Tjellström et al. 2008) and nitrogen limitation (Gaude et al. 2007). Under these conditions, phospholipids throughout the cell are remobilized to the plastid and converted to galactolipids before export and accumulation in extraplastidic membranes. This process involves a major flux of lipids through the chloroplast envelope (Block et al. 2007). Increased stromule frequency has been observed during phosphate limitation (Vismans et al. 2016). Although further

research is necessary, this suggests at least a preliminary correlation between times of increased lipid trafficking and the occurrence of stromules.

Despite a potential role for lipids in altering the behavior of the plastid envelope, such changes do not seem to influence the long-term efficiency of galactolipid accumulation. Mutants with altered strigolactone signaling and synthesis show either high or low stromule frequency under phosphate-limiting conditions. However, galactolipid synthesis and accumulation during phosphate limitation appear unchanged in these mutants (Vismans et al. 2016). This argues against the postulated role for stromules in promoting lipid exchange, but does not negate the potential for lipid trafficking to influence plastid shape or the importance of MCSs in facilitating this exchange.

## 9.7 Modulation of Plastid Form by Extraplastidial Membranes

Just as the structure and composition of a plastid appear to affect its function and morphology, the surrounding cellular environment must be considered a sculptor of plastid shape. It has been suggested that MCSs between the ER and many organelles, including the plastid, create an extensive network that extends to the furthest reaches inside a cell. Current views of MCSs suggest protein-mediated hemifusion of the outer leaflets of two organelle membranes as a leading model of MCS formation (Prinz 2014; Pérez-Sancho et al. 2016). MCSs between the ER and mitochondria in animal and yeast systems have been the most extensively studied. Mitochondria-associated membranes (MAMs) found on the ER contain specific lipases required for mitochondrial lipid synthesis (Vance 1991), supporting their potential role in lipid exchange. Although no proteins that are associated with MCSs between plastids and the ER have been conclusively identified to date, evidence for the connection is strong and MCSs (also termed plastid-associated membranes or PLAMs) are a frequent point of discussion in the study of metabolite trafficking. Transmission electron microscopy images show that the ER is closely associated with plastids (McLean et al. 1988; Whatley et al. 1991), and membrane continuities between the chloroplast outer envelope membrane and the ER have been suggested in some species (Crotty and Ledbetter 1973). Plastids are normally seen embedded in a cage of ER tubules (Schattat et al. 2011a). Early work suggested a physical attachment between these two organelles; for example, Stumpf et al. (1963) observed that vesicular spheres remained stuck to the outer membrane of isolated plastids. A more recent study used laser tweezers to highlight the difficulty of separating plastids from the ER: When protoplasts containing a GFP-tagged ER lumen protein were ruptured, plastids were pulled away from the cell and a considerable amount of force was needed to disassociate the ER from the plastid (Andersson et al. 2007). This evidence all suggests the presence of PLAMs and, although lipid trafficking is their primary proposed role (Wang and Benning 2012), they may also play a part in plastid movement and pleomorphy.

The movement and rearrangement of the ER cage that surrounds the plastid correlates with plastid movement and with the extension of stromules from chloroplasts. Furthermore, branching of stromules always occurs at an angle that correlates with ER polygon junctions (Schattat et al. 2011a, b). This led to the suggestion that the localized force exerted on a plastid body at MCSs as a result of ER rearrangement might account for stromule extension. Although this ER–plastid interaction could partially explain the ability of plastids to produce stromules, they are not likely to account entirely for the phenomenon. The ER is almost constantly in motion and therefore consistently exerts a force on the plastid. However, a fairly small population of plastids exhibit stromules at any given moment. The production of stromules probably results from the correlation of internal factors (such as membrane lipid profiles) and external physical forces. In addition to the ER, the actin cytoskeleton has also been implicated in stromule extension through the exertion of physical forces by myosin motors, and this system is known to play an important role in plastid positioning and movement.

## 9.8 Involvement of Plastid Positioning and the Actin Cytoskeleton in Plastid Morphology

Just as a plastid's morphology and metabolic status are not fixed traits, the physical location of a plastid within the cell is variable. Plastid movement is best understood in relation to the accumulation and avoidance responses to blue light (Sakai et al. 2001; Kagawa et al. 2004). This phenomenon was first observed by Böhm (1856), who saw that the arrangement of chloroplasts in a leaf changed depending on lighting conditions. Today, it is known that chloroplasts accumulate on the irradiated side of the cell under low intensity blue light, but move away from the light source under high intensity blue light (Sakai et al. 2001; Kagawa et al. 2004). This response has been proposed to promote the proper positioning of chloroplasts for optimal energy capture during low light conditions, while minimizing photo-oxidative damage during high light conditions (Takahashi and Badger 2011; Kasahara et al. 2004). Blue light photoreceptors known as phototropins (PHOT) are responsible for regulating chloroplast movement in response to blue light. In *A. thaliana*, PHOT1 and PHOT2 both contribute to the accumulation response, whereas PHOT2 alone appears necessary for the avoidance response (Jarillo et al. 2001; Kagawa et al. 2001; Sakai et al. 2001).

Plastid movement and positioning are actin-dependent phenomena. Actin-disrupting drugs inhibit light-induced chloroplast movement responses in the aquatic plant *Lemna trisula* L. (Malec et al. 1996) and in *A. thaliana* (Paves and Truve 2007). An apparent association between plastids and long actin filaments has been suggested (Kandasamy and Meagher 1999; Anielska-Mazur et al. 2009); myosins appear to localize to plastids in *Zea mays* L. (Wang and Pesacreta 2004), *N. benthamiana* (Sattarzadeh et al. 2009), *L. trisula* (Malec et al. 1996), and

*A. thaliana* (Wojtaszek et al. 2005; Krzeszowiec et al. 2007). Chloroplasts are also enmeshed in a cage of short actin filaments (Kwok and Hanson 2004a; Kadota et al. 2009) that is implicated in chloroplast positioning, as the filaments are seen to rearrange during chloroplast movement (Kong et al. 2013). An *Arabidopsis* mutant, *chloroplast unusual positioning1* (*chup1*; Kasahara et al. 2002; Oikawa et al. 2003), lacks the perichloroplastic actin cage and does not exhibit a blue light avoidance response (Kadota et al. 2009). The CHUP1 protein is found on the plastid outer envelope and is thought to simultaneously associate with the actin cytoskeleton and anchor the plastid directly to the plasma membrane (Oikawa et al. 2008). Interestingly, the *chup1* mutant is reported to possess a high stromule phenotype, suggesting that CHUP1 and the perichloroplastic actin cage influence plastid shape by preventing stromule formation (Caplan et al. 2015). What implications this has for the shape of plastids during relocation remains unexplored.

The potential roles of actin-associated CHUP1 and the cage of periplastidic actin in restricting stromule formation are somewhat at odds with observations suggesting that the extension of stromules is actin dependent. The alignment of stromules with actin filaments has been reported (Kwok and Hanson 2004a), and stromules in plants treated with actin-disrupting drugs show reduced frequency, shorter morphology, and lose their appearance of tension (Kwok and Hanson 2003; Natesan et al. 2009). Similarly, RNAi knockdown of myosin XI-2 also disrupted normal stromule behavior. Interestingly, actin does not appear to play a role in the formation of smaller protrusions (Holzinger et al. 2007b). The actin-myosin theory of stromule extension is similar to that of the ER-MCS theory, with myosin-based attachment points on the plastid envelope membrane acting as tethering points within the cell and assisting in stromule extension (Hanson and Sattarzadeh 2011). It is difficult to favor either of the physical force stromule formation models because the actin cytoskeleton and the ER are irrevocably interconnected. ER rearrangement is dependent on the actin cytoskeleton, and any disruption of the actin-myosin system impairs ER movement (Peremyslov et al. 2008, 2010; Ueda et al. 2010). Conversely, drugs known to influence ER morphology, such as Brefeldin A, alter the behavior of the actin cytoskeleton (Hörmanseder et al. 2005; Takác et al. 2011). Therefore, it is difficult to assess whether the production of stromules is dependent on physical connections to the ER, actin, or both structures.

The focus so far has been primarily on the rapid formation of protrusions or stromules from a plastid; however, on a larger timescale, other factors can influence the morphology of the plastid population seen within a cell or tissue.

## 9.9 Changes in Plastid Shape During Plastid Division

New plastids arise from the division of existing plastids in a cell. The process involves constriction of the plastid mid-region and has been well studied in algae (West and Starkey 1915; Green 1964; Bisalputra and Bisalputra 1970), nonvascular

plants (Lander 1935; Gantt and Arnott 1963; Whatley 1974), and vascular plants (Juniper and Clowes 1965; Possingham and Saurer 1969; Lyndon and Robertson 1976; Pyke et al. 1994). During the early stages of leaf development, numerous chloroplasts with midpoint constrictions can be observed as a transitory stage in the increase of chloroplast number during leaf expansion, providing a period where plastid morphology is distinctly different from that in a mature leaf (Possingham and Saurer 1969; Boasson et al. 1972; Platt-Aloia and Thomson 1977; Boffey et al. 1979). Prior to division, the mid-region of a dividing plastid becomes narrow and almost tubular, forming an isthmus (Leech et al. 1981). An electron dense, "fuzzy plaque" or ring can often be observed at such constrictions (Mita et al. 1986; Mita and Kuroiwa 1988; Hashimoto 1986, 1997). The process of division appears to be ubiquitous among plastid types, as these division rings are visible on dividing proplastids (Suzuki and Ueda 1975; Chaly and Possingham 1981), chloroplasts (Hashimoto 1986), and amyloplasts (Luck and Jordan 1980).

In the process of investigating plastid division, it has become apparent that aberrant division can have severe effects on plastid morphology. Visual screens for *A. thaliana* L. mutants with altered plastid number and shape revealed a number of mutants, including those of the *accumulation and replication of chloroplasts (arc)* family (Pyke and Leech 1991; Pyke et al. 1994; Robertson et al. 1996). These mutants show reduced chloroplast number, changes to chloroplast size and, interestingly, an alteration in the production of stromules. This change in stromule production is not a result of changed plastid density. Although an indirect or direct role for the division machinery in stromule formation is postulated, it needs further investigation (Holzinger et al. 2008).

## 9.10 Conclusions

The plastid is a very important organelle within the plant cell, and its role in a wide variety of biochemical pathways is well established. Its variable form has long been known, but only in recent decades has plastid morphology become an active point of focus in plastid biology. Changes to plastid shape are influenced by internal factors such as the accumulation of inclusions and the structure of internal membranes; they may also be influenced by changes in the lipid profile of the plastid envelope. External factors are also important in any discussion of plastid morphology, as both the ER and the actin cytoskeleton are thought to be in close physical association with the plastid through MCSs and myosins, respectively. The dynamic interactions between these elements are capable of influencing plastid movement and shape. Whether the formation of stromules or other alterations to plastid shape change the effectiveness of the organelle's function is currently unknown, but shape changes certainly allow greater outreach and a possible increase in interactivity between the organelle and neighboring cytoplasm.

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# Chapter 10
# Communication Within Plant Cells


**Bratislav Stankovic and Eric Davies**

**Abstract** This chapter is concerned with the intracellular signaling events that take place in individual plant cells. It summarizes the known intracellular signaling events, cascades, molecular and cellular participants, receptors, transducers, and effectors; as well as their connectivity, interplay, and crosstalk. First, we review the signaling anatomy and physiology of a plant cell, describing the molecular and cellular components that are involved. Then, we provide an overview of the events taking place from perception to attenuation in a variety of contexts. We highlight some intracellular signaling components that are unique to plants. Finally, we use case studies of several types of plant cells and of several types of stimuli that trigger signaling events and result in cellular responses. The analysis of signaling pathways and networks has become an essential tool for understanding cellular functions. The presence of numerous components, their degree of interconnectivity, and their dynamic spatiotemporal redistribution all contribute to the complexity of signaling pathways. We suggest that unveiling the cellular and molecular details of the myriad of intracellular signaling processes is essential for a complete understanding of whole-plant physiology.

## 10.1 The Stimulus/Signal Conundrum

Many authors use the words "stimulus" and "signal" interchangeably, but we prefer the concept of the stimulus being that which is emitted, and the signal being that which is perceived. Accordingly, stimuli are emitted by both nonbiological entities (e.g., light, heat, cold, water, nutrients, gravity, pressure, touch, and

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electromagnetic fields) as well as biological entities (e.g., large herbivores, insects, nematodes, bacteria, and fungi). In order for a stimulus to evoke a response, it must be perceived, with stimulus perception being a purely biological phenomenon. Once a stimulus is perceived, it becomes a signal that can then evoke generation of a new (second) stimulus, which, once perceived, becomes a second signal (second messenger) that initiates a signal transduction pathway. This concept can be understood by considering Newton's apple. When the apple fell, it had experienced a nonbiological stimulus (presumably a gust of wind), which evoked the nonbiological response of falling, as a result of another nonbiological stimulus (gravity). However, when the apple landed on Newton's head it was perceived by a biological entity (Newton's pain receptors) and evoked a biological response such as "Ouch. That hurt!" He was an English gentleman.

Both stimuli and signals are forms of information transfer and, as suggested earlier (Vian et al. 2015), we envisage a stimulus as a packet of information emitted (by either abiotic or biotic factors), whereas a signal is a packet of information as perceived by an organism. In turn, this can evoke a downstream response of generating another stimulus, which acts as a signal, and so on.

Stimuli external to the cell under consideration may come from the environment, including from both the nonbiological and the biological agents listed above or from an adjacent cell, and stimuli/signals generated in that cell can be transmitted to other cells or to the environment. A new and developing area in plant sciences is "plant neurobiology," in which scientists explore how plants perceive stimuli within their environment and convert them into internal electrochemical ("plant neurobiological") signals. The perceived and transmitted signals permit rapid modifications of physiology and development that help plants adjust to changes in their environment (Barlow 2008). This chapter focuses on stimuli from without (environment, adjacent cells), stimuli from within (intracellular signaling), and stimuli emitted into the environment and adjacent cells.

## 10.2 The Signaling Anatomy and Physiology of a Plant Cell

The body plan of higher plants is controlled by a combination of clonal fate and positional information that is provided by local signals. In plants, much as in animals, cells are in constant communication with one another. Plant cells communicate to coordinate their activities in response to the changing conditions of light, dark, temperature, and nutrient availability that guide the plant's cycle of growth, flowering, and fruiting. Plant cells also communicate to coordinate what goes on in their roots, stems, leaves, and flowers. This section considers how plant cells signal to one another and how they respond to the perceived signals. In particular, we look at how the receptors and intracellular signaling mechanisms involved in plant cell communication differ from those used by animals.

Signal transduction pathways link signal reception to response, referring to the transmission of a molecular signal in the form of a physicochemical modification

(e.g., by a change in electric potential, membrane/cytoskeleton conformation, or recruitment of protein complexes) along a signaling pathway that ultimately triggers a biochemical event in the cell. Plant perception refers to the ability of plants to sense and respond to the environment and to adjust their morphology, physiology, and phenotype accordingly (Trewavas 2005). Within the cell, signaling occurs at a myriad of places. Signaling occurs everywhere: circulating around the cell, in cytoplasmic streaming, between organelles (e.g., nucleus to cell wall), within organelles (e.g., cell wall, cytoplasmic membrane, nucleus, mitochondria, plastids, endoplasmic reticulum, and especially the cytoskeleton), and within molecules. Numerous components in a signaling pathway are responsible for the signaling process. Each component is classified according to the role it plays with respect to the initial stimulus. For example, ligands are typically termed "first messengers," whereas receptors (both plasma membrane-bound and intracellular) are termed "signal transducers," which then activate primary effectors in a typical signaling cascade. Signaling is accomplished through different modes of transduction/conveying information.

Plants often use cell surface receptors belonging to the families of histidine kinases or receptor-like serine/threonine kinases to sense stimuli and to trigger responses through intracellular phosphorylation cascades. First messengers are the signaling molecules that reach the cell from the extracellular/apoplastic space and bind to their specific receptors. Second messengers are the substances that enter the cytoplasm, or are already present in the cytoplasm, and act within the cell to trigger a response. In essence, second messengers serve as physicochemical relays from the plasma membrane to the targeted location, thus carrying out intracellular signal transduction. In higher plants, reversible protein phosphorylation is a prevalent mechanism in the signal transduction pathways. It connects signal perception mechanisms to responses, and also provides crosstalk and interconnection of regulatory components in signaling networks. Indeed, the largest proportion of the higher plant's genome is devoted to codes for protein kinase and protein phosphatase genes (Bögre 2007). Specific responses to the variety of stimuli are accomplished by engagement of particular cellular components and molecules. Intracellular regulatory pathways can also serve as receptors for plant hormones (e.g., auxin, gibberellin, ethylene, cytokinin, jasmonate, brassinosteroids, and peptide hormones); these often help to strengthen plasticity.

The cytoskeleton has an important role in mediating the plant cell's response to biotic factors by acting as a regulator and target of biotic interactions in plants, as a scaffold for tethering transport, and for targeting mRNAs to specific cellular microdomains. Remodeling of the plant cytoskeleton is instrumental in achieving structural cellular responses to external stimuli. Actin and microtubule arrays participate in signaling cascades initiated at the plasma membrane, enabling adaption to environmental factors. For example, changes in cytoskeletal organization facilitate signaling of the presence of symbionts or pathogens on the plant (and thus cellular) surface. The cytoskeleton plays a role in cytoplasmic aggregation, as seen in the response to mycorrhizal fungi, the establishment of symbiotic relationships in rhizobia, the cellular response to inoculation, the self-incompatibility response

during fertilization, and the response to bacterial and viral infections (Takemoto and Hardham 2004).

Intracellular signaling complexity comprises elaborate control circuitry in the form of signaling complexes and networks. One of the first examples of complex signaling networks was provided by one of the authors of this review (Davies 1987), who described the interwoven pattern of plant responses to wounding. Appropriate network behavior and dynamics are accomplished via robust feed-forward and negative feedback controls, where redundancy is necessary for reliability. The strength of connections between the network elements is dynamic and responsive to the environmental context (Trewavas 2002).

Downstream in the signaling cascades are the molecular consequences of the initial signal perception. These include molecular changes evoked by intracellular signals on transcription, translation, cytoskeleton actin, protein–mRNA interactions, and metabolic alterations. Second messengers (e.g., calcium, cyclic nucleotides) can act in local microdomains, where ephemeral protein complexes are often formed, such as at the sites of signal perception and signal transduction. These protein complexes may include calcium-binding proteins complexed with ion channels and calcium-dependent protein kinases bound to the cytoskeleton.

As we develop our understanding of the plethora of components of plant signaling, we discover that many of them are united in diversity. For example, the reactive oxygen species (ROS)-mediated mitogen-activated protein kinase (MAPK) pathway is both strikingly similar and different in the plant responses to abiotic and biotic stresses. ROS activate a similar MAPK in response to different environmental stimuli, showing different downstream targets with different and specific responses (Jalmi and Sinha 2015). In addition, the mechanosensitive (MS) ion channels, which are a common mechanism for perceiving and responding to mechanical force, come in three families in plants: the MscS-like (MSL), the Mid1-complementing activity (MCA), and two-pore potassium (TPK) families. Channels from these three families vary widely in structure and function, localize to multiple cellular compartments, and conduct different ions (chloride, calcium, and/or potassium). However, these channels probably represent only a fraction of the MS ion channel diversity in plant systems (Hamilton et al. 2015).

Distinguishing themselves from other eukaryotes, plants have certain unique signaling components. For example, plant histidine-specific protein kinases are structurally distinct from other protein kinases and function as part of a two-component signal transduction mechanism: A phosphate group from ATP is first added to a histidine residue within the kinase, then transferred to an aspartate residue on a receiver domain on a different protein or on the kinase itself, thus activating the aspartate residue (Wolanin et al. 2002). Plant histidine kinases are involved in crosstalk that exists between hormones and stress responses. Despite their structural diversity, the histidine kinases exhibit functional redundancy. Several sensory histidine kinases having a cytokinin-binding CHASE (cyclases/histidine kinases associated sensory extracellular) domain, transmembrane domain(s), transmitter domain, and receiver domain are involved in cytokinin and ethylene signaling. On the other hand, some of the sensory histidine kinases perform as

osmosensors, clearly indicating a possible crosstalk between hormone and stress-responsive cascades (Nongpiur et al. 2012).

## 10.3 From Perception to Attenuation: Signal Transduction "Outside to Intracell"

### *10.3.1 Developmental Signaling*

Plant development depends on both intracellular and intercellular communication. Intracellular signals can arise endogenously in response to normal environmental stimuli, that is, typical/steady-state intensity conditions that occur on a daily basis (Van Norman et al. 2011). For example, developmental signal transduction occurs in the following: (1) vegetative phase (as homeostasis and organogenesis, growth, cell enlargement, and cell division); (2) regenerative phase (flowering) and fertilization; (3) plant growth (e.g., in response to developmental action of phytohormones, biosensors, and nitrogen fixation); (4) plant immunity; (5) fruit formation; and (6) plant ageing, followed by apoptosis.

Many aspects of plant growth and development are regulated by networks of intracellular signaling mechanisms rather than by linear signal transduction pathways, thus rendering plants particularly attractive for dissecting crosstalk and signal specificity mechanisms (Giraudat and Schroeder 2001). For example, whole-plant homeostasis is maintained with the help of endogenous signals, which arise within plant cells and are continually modulated in response to environmental changes. Homeostasis is of considerable importance to plants because they are trapped unmoving within their changing surroundings. Homeostasis is chiefly influenced by the levels of intracellular plant hormones. In maintaining homeostasis, various signaling peptides are also important signaling players, whose roles are intricately interwoven with the "classical" hormones to regulate plant growth and development as well as responses to the environment. Homeostatic signaling peptides include (1) plant natriuretic peptides (PNPs), which modulate ion channels and water uptake; (2) phytosulfokines (PSKs); and (3) rapid alkalinization factors (RALFs) (Gehring and Irving 2012). Other signaling peptides, such as systemin, are known to be involved in the plant signaling of wounding-induced defense responses (Ryan and Pearce 1998).

The calcium ion ($Ca^{2+}$) is recognized as a crucial second messenger in plant signaling. One of the earliest events following perception of environmental change is intracellular variation in free calcium concentration. These calcium variations differ in their spatiotemporal characteristics (subcellular location, amplitude, kinetics) with the nature and strength of the stimulus, and are therefore considered as signatures encrypting information from the perceived stimulus. This information is believed to drive a specific response by decoding via calcium-binding proteins. The number of calcium sensor proteins is vast; it is estimated to exceed 250 in

*Arabidopsis*. The calcium-sensing proteins are represented by different families (calcineurin-B-like proteins, calmodulin, and calmodulin-like proteins; calcium-dependent protein kinases; and calcium and calmodulin-dependent protein kinases). The $Ca^{2+}$-induced conformational changes probably increase their interaction affinity to downstream effectors (Ranty et al. 2016).

Signaling in plants may be related to the existent metabolism and accompanying energy pools. Plant cells release adenosine triphosphate (ATP) into their extracellular matrix, which then modulates the rate of cell growth in diverse tissues. For example, in *Arabidopsis thaliana*, the extracellular ATP concentration is controlled by two closely related apyrases, APY1 and APY2. Suppression of APY1 and APY2 is linked to severe growth inhibition, through altered expression of genes involved in biotic stress responses, specifically including genes regulating cell wall composition and extensibility. These transcriptional changes are ultimately translated into metabolic alterations, including wall lignification and decreased methyl ester bonds. Apparently, apyrases can play important roles in the signaling steps that link biotic stresses to plant defense responses and growth changes (Lim et al. 2014).

The role of sucrose as a signaling molecule in plants is still poorly understood. The best studied sucrose signaling-driven processes affect general plant metabolism and take place in different tissues and organs simultaneously. Yet others occur in meristems, giving rise to changes in developmental patterns. These involve metabolic processes, such as induction of fructan or anthocyanin synthesis. Sucrose concentration in plant tissues is correlated to light intensity and is inversely related to temperature. Accordingly, exogenous sucrose supply often mimics the effect of increased light or cold. However, many exceptions to this rule seem to occur because of interactions with other signaling pathways (Tognetti et al. 2013).

Lipid signaling plays diverse roles in various cellular and physiological processes and rightfully deserves entire books devoted to the topic (Wang and Chapman 2013). Membrane lipids provide both the structural basis for cell membranes and a rich source of cellular mediators that regulate many aspects of plant development and environmental interactions. Several classes of lipids and their related metabolites are known to be involved in signaling, including phosphatidic acid, oxylipins, phosphoinositides, sphingolipids, free fatty acids, lysophospholipids, *N*-acylethanolamines, and oxidatively modified galactolipids. Yet, identifying lipid-interacting proteins and translating the milieu of lipid metabolite changes in cells into the mechanisms for regulation of physiological processes in plants remains a formidable challenge (Wang and Chapman 2013).

The transition from vegetative growth to flowering is regulated by sensing the seasonal changes in environmental parameters, such as day length (or night length). This process chiefly proceeds through a genetically defined intracellular signaling cascade known as the photoperiodic pathway. Anticipation of seasonal change is perceived through changes in day length, which are the causal agent of seasonal climate (Golembeski and Imaizumi 2015). Recent studies have highlighted the key role of the FLOWERING LOCUS T (FT) protein in regulating floral induction. The FT protein is the "florigenic" signal that is produced in leaves in response to

inductive day lengths and traffics through the phloem to initiate flowering at the shoot apex (Giakountis and Coupland 2008).

### *10.3.2 Signaling Induced by Exogenous Stimuli*

Most intracellular signals are elicited from factors outside the individual cell, either from other cells or from the environment. These factors include stresses, either abiotic (drought, salt, poor nutrients, mechanical forces, gravity, temperature extremes, electromagnetic fields, wounding, light, heavy metals/detoxification, etc.), or biotic (other plants, animals/insects, grazing, viruses, and pathogen attacks being nonlimiting examples). Being sessile organisms and rooted in one place, plants must respond to changes in exogenous (abiotic) stimuli presented as dynamic environmental conditions and stresses. Indeed, plants exhibit distinct changes in gene expression, metabolism, and physiology in response to different environmental stress conditions. Accordingly, it is safe to presume that plant cells must be capable of sensing various environmental signals. The core abiotic stress-signaling pathways, in response to salt, drought, and the stress hormone abscisic acid (ABA), largely depend on the SnRK (SNF-related kinase) family of protein kinases; these kinases are related to the yeast SNF1 (sucrose nonfermenting 1) and mammalian AMPK (AMP-activated protein kinase), which are key sensors of cellular energy status. This further suggests that abiotic stress signaling in plants evolved from energy sensing. However, despite much effort, the abiotic stress sensors in plants remain elusive, and only a few putative sensors have thus far been identified (Zhu 2016).

The signal transduction pathways form complex networks that control plant responses to the environment. The identification and characterization of individual functional units involved in signaling cascades has facilitated our understanding of the flow of information in response to a given stimulus. Functional signaling units involve, without limitation, downstream signaling proteins, ion channels, enzymes such as protein kinases and protein phosphatases, G-protein signaling components, transcription factors, microRNAs, inositol phosphates, ROS, and intracellular $Ca^{2+}$ concentration (Pandey et al. 2016). Stimulus-induced oscillations in cytosolic free calcium encode information that is used to specify the outcome of the final response. For example, calcium oscillations are involved in the control of guard cell turgor, Nod factor signaling, and pollen-tube growth (Giraudat and Schroeder 2001). The responses to various exogenous stimuli are outlined next.

**Cold, Drought, and Salt**

Cold (low temperature), water stress (drought), and high salinity are complex environmental stimuli that possess many common attributes. For example, salt stress includes both an ionic (chemical) component and an osmotic (physical) component (Xiong et al. 2002). Both salt and drought stress signal transduction consists of ionic and osmotic homeostasis signaling pathways, detoxification (i.e.,

damage control and repair) response pathways, and pathways for growth regulation (Zhu 2002). Acting as the selective barrier between living cells and their environments, the plasma membrane plays a key role in the perception and transmission of external information. Upon osmotic stress, changes in phospholipid composition are detected in plant cells. During exposure to stress, the major role of phospholipids is probably to serve as precursors for the generation of second-messenger molecules. Relevant cleaving enzymes are the phospholipases A2, C, and D, but the most studied is phosphoinositide-specific phospholipase C (PI-PLC). PI-PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) upon activation. PIP2 regulates cytoskeleton–plasma membrane adhesion and is itself a signal involved in recruitment of signaling complexes to specific membrane locations, and in their assembly (Xiong et al. 2002). Osmotic stress activates several protein kinases, including mitogen-activated kinases, which may mediate osmotic homeostasis and detoxification responses. Signaling through the SOS (salt overly sensitive) pathway, a $Ca^{2+}$-responsive SOS3-SOS2 protein kinase complex controls the expression and activity of ion transporters such as SOS1. A number of phospholipid systems are activated by osmotic stress, generating a diverse array of messenger molecules and abscisic acid biosynthesis (Zhu 2002).

**Gravity**

Since the early days of plant molecular biology, signaling in gravitropism has attracted the attention of plant biologists. Molecular evidence has provided support for two long-surviving hypotheses about the mechanism of gravitropism: the starch–statolith hypothesis and the Cholodny–Went hypothesis. It appears that movement of amyloplasts along the gravity vector within gravity-sensing cells in roots and shoots is the most likely trigger of subsequent intracellular signaling. Several possible events leading from this signaling to differential auxin distribution within the sensing cells (and subsequent differential curvature and growth) have been suggested (Morita and Tasaka 2008).

**Light**

Signaling and the regulation of gene expression by light has been an active area of research for a long time. Subjects of study include families of photoreceptors (phytochromes, cryptochromes, and phototropins), the light signaling genes downstream of the photoreceptors, and the resulting growth control mechanisms. The accompanying signal transduction mechanisms involve receptor dimerization, changes in cytoplasmic $Ca^{2+}$ concentration, 14-3-3 proteins, and proton pump stimulation. Crosstalk between photoreceptors sensing red light and blue light (phytochromes and cryptochromes, respectively) occurs at all stages of plant growth and development. The study of light signaling in plants has reached a new level of sophistication with the availability of multiple microarray datasets (Spalding 2003). Between light absorption by photoreceptors and the physiological and developmental responses lies a web of interacting factors and interacting pathways, which are either directly involved in, or otherwise impinging upon, light signal transduction (Eckardt 2004).

**Nutrients**

Nutrient metabolism must be tightly coordinated to sustain optimal growth and development. Because plants often encounter nutrient deficiency in their surrounding environments, and also because they are sessile, they have developed sophisticated strategies to cope with nutritional stress. The strategies include an array of biochemical, physiological, and developmental responses. The intimate crosstalk among the various nutrients suggests the existence of some common signaling components that are involved in regulating plant responses to different nutrient stresses. Sucrose regulates plant deficiency responses to multiple nutrients and is part of a general response to nutrient deprivation (Lei and Liu 2011). However, the molecular identities of many signaling components that are involved in regulating plant responses to different nutrient stresses remain elusive. In particular, the balance of cellular carbon (C) and nitrogen (N) is important. However, despite exciting progress toward dissecting the C/N balance response regulatory network and the crosstalk of C and N pathways, little is known regarding the signal sensed or which pathway operates in the complex C/N balance response (Zheng 2009).

**Wounding**

Wounding activates cellular mechanisms directed to healing and defense. Some of the physicochemical components involved in transducing wound signals (ion concentration fluctuations, changes in electric potentials, mechanotransduction, chemicals) also function in signaling other plant defense responses, suggesting that crosstalk events regulate temporal and spatial activation of different defenses (León et al. 2001). Identifying and characterizing receptors for wound signals is likely to be an important focus of future research in the field of plant responses to wounding.

**Electromagnetic Fields**

High frequency nonionizing electromagnetic fields (EMF) are increasingly present in the environment and constitute a genuine environmental stimulus that is able to evoke specific responses in plants. These responses share many similarities with those observed after a stressful treatment. Indeed, numerous metabolic activities (ROS metabolism, α- and β-amylase, Krebs cycle, pentose phosphate pathway, chlorophyll content, terpene emission, etc.) are modified, gene expression (e.g., calmodulin, calcium-dependent protein kinase, and proteinase inhibitor) is altered, and growth (stem elongation and dry weight) is reduced after low power EMF exposure. These changes occur not only in the tissues directly exposed, but also in systemically distant tissues (Vian et al. 2016).

**Pathogens**

The plant's surveillance system for pathogen attack is based on early recognition of the invading organism(s) and the subsequent activation of appropriate defense mechanisms. For example, endogenous cyclic AMP is involved in plant defense responses against fungal (*Verticillium dahlia*)-secreted toxins, by regulating the production of the known defense-related signal salicylic acid and the subsequent activation of a defense pathway (Jiang et al. 2005).

## 10.4 Specific Cell Types

### *10.4.1 Signaling in Dividing Cells*

Intensive signaling occurs in dividing plant cells, in which the preprophase band (PPB) is formed by a cortical array of microtubules. The PPB appears in G2 phase and prophase, and predicts the future division site (i.e., location of the future cell plate insertion). The PPB disappears at the prophase/prometaphase transition stage, but it leaves information in some as-yet-unidentified form at the site of division and future cell wall localization. Several kinds of signaling molecules are reported to occur in PPBs, but their roles are poorly understood (Mineyuki 1999). It is possible that pressure is exerted by the PPB locally on the plasma membrane, which then causes mechanosensitive calcium ion channels to start pumping $Ca^{2+}$ in, which in turn stimulates Golgi vesicles to coalesce (Davies et al. 1996).

### *10.4.2 Signaling in Guard Cells*

Guard cells are pairwise located in the epidermis of plant leaves and stems, where they surround stomatal pores. The stomatal pore openings allow carbon dioxide influx for photosynthetic carbon fixation, and also enable water loss to the atmosphere via transpiration. In these competing processes, plants lose most of their absorbed water via transpiration. Signal transduction mechanisms in guard cells integrate a multitude of different stimuli (light signals, water status, $CO_2$, temperature, hormonal stimuli, and other environmental conditions) to modulate stomatal aperture for regulation of gas exchange and for plant survival under diverse conditions (Schroeder et al. 2001). Guard cells have thus become a well-developed system for dissecting early signal transduction mechanisms and for elucidating how individual signaling components and mechanisms can interact within a signaling network in a single cell. Of particular significance in this signaling process is the role of the hormone abscisic acid, which triggers closing of stomatal pores.

### *10.4.3 Signaling in Vascular Cells*

Plant vascular cells are formed under a well-defined three-dimensional plant differentiation program, which has unique features, including the cell-death program. Although vascular cells usually differentiate at predicted positions and at a predicted time to form a specific vascular pattern, the arrangement of the vascular network can be altered by local signals or in response to environmental stimuli. Crosstalk exists between plant hormones, functioning as intercellular signals, and their endogenous biosynthetic processes in distinct vascular cells. This ensures the

activation of procambial cells and their differentiation into various vascular cells with distinct functions. Crucial roles in the formation and/or maintenance of vascular cells are ascribed to cytokinin, gradual expression of the HD-ZIP-III (homeobox leucine-zipper protein) homeobox genes, microRNAs, brassinosteroids, and xylogen. Unique processes in tracheary element formation include the development of patterned secondary walls and programmed cell death. Vascular cell polarity is accomplished through asymmetrical intracellular signaling pathways that establish this polarity (Fukuda 2004).

### *10.4.4 Signaling in Pollen*

Fertilization is a key life event for sexually reproductive plants. A unique feature of sexual reproduction in angiosperms is the pollen tube, which is a tubular tip-growing cell germinated from a pollen grain. The highly polarized process of tip growth requires intensive exocytosis at the tip, which is supported by a dynamic cytoskeleton and vesicle trafficking. Different signaling pathways and networks coordinatively modulate the rapid growth of pollen tubes by regulating cellular activities such as actin dynamics, exocytosis, and endocytosis. The homeostasis of key signaling molecules is crucial for proper elongation of the pollen tube tip; it is fine-tuned by both positive and negative regulation (Guan et al. 2013). In addition to the major signaling pathways, other signals are involved in the regulation of pollen tube growth and the guidance of its journey during fertilization, including numerous peptides required for micropylar pollen tube guidance (Kanaoka and Higashiyama 2015).

### *10.4.5 Signaling in Root Hair Cells*

The process of nodule development is unique to plants. It is the result of rhizobia–legume symbioses and governs the (de)formation of root hairs. The onset of nodule development is determined by the exchange of chemical compounds between the microsymbiont and the leguminous host plant. Lipo-chitooligosaccharidic nodulation (Nod) factors, secreted by rhizobia, belong to this set of signal molecules. Nod factors consist of an acylated chitin oligomeric backbone with various substitutions at the (non)reducing terminal and/or nonterminal residues. The Nod factor-related signals are perceived using a symbiosis receptor-like kinase (SYMRK) and various transmembrane Nod factor receptor (NFR) kinases. The sequence of responses directly downstream of Nod factor perception includes changes in $Ca^{2+}$ fluxes, membrane potential, and pH (as intra- and extracellular alkalinization). The targeted physiological processes include the formation and deformation of root hairs, early nodulin gene expression, and nodule primordia formation. A correct chemical structure is required for induction of a particular plant response,

suggesting that Nod factor–receptor interaction(s) precede a Nod factor-induced signal transduction cascade (D'Haeze and Holsters 2002). Interestingly, the plant-parasitic root-knot nematodes (RKN) invoke a spatiotemporal cytoskeletal response, subcellular reorganization, and root hair deformation identical to that seen in the plant cell response to Nod factors (Weerasinghe et al. 2005).

## 10.5 Prospects

Even though signal transduction is far too complex to be carried out by a linear backbone, researchers continue to chip away at the plant's unknown signaling mechanisms. Substantial advances in our understanding of many plant signal transduction pathways have been made through interdisciplinary studies. It appears that many of the central signaling mechanisms in plants deviate from animal cell paradigms. In a signal transduction context, the external stimuli are perceived by cells via a sensor or receptor that is often, but not exclusively, situated on the plasma membrane (plasmalemma). Interaction of the stimulus with its corresponding receptor induces a cascade of intracellular signaling processes, which culminate in responses at the cellular and, ultimately, whole-plant level. The signal transience is often accompanied by rapid changes in protein synthesis and transcript accumulation (Davies 1987). Some signaling events are very rapid, occurring within seconds or minutes; they often involve changes in the activities of ion channels and enzymes. Longer-term responses often involve transcriptional and translational changes, and might be integral facets of the short-term responses. Typical intracellular signaling responses involve the generation of second messengers, which are signaling molecules whose concentrations and/or conformations are transiently altered in response to an external signal. Their biological activity results from their interactions with downstream signaling components, which are primarily proteins. The challenges in this exciting field include understanding how the spatiotemporal calcium oscillations are generated and decoded.

The identification and heterogeneous distribution of signaling molecules and switches in organelle-specific subcompartments is another exciting area for future research that will improve our understanding of how different signaling networks in plant cells are integrated. Interestingly, it has been shown in animal cells that the morphologically heterogeneous Golgi apparatus is endowed with $Ca^{2+}$ pumps, $Ca^{2+}$ release channels, and $Ca^{2+}$ binding proteins. The Golgi is now thought to participate in determining the spatiotemporal complexity of the $Ca^{2+}$ signal within cells via heterogeneity in terms of both $Ca^{2+}$ handling and selective reduction of $Ca^{2+}$ concentration (Pizzo et al. 2011). Possible analogous processes in plant cells are still poorly understood.

Plants constitute an outstanding model for the study of environmentally induced signaling processes, because their architecture (high surface area to volume ratio) optimizes their interaction with the environment (Vian et al. 2016). In addition, plants cannot escape the environment and therefore they must constantly monitor

it. It is also of major interest to unravel the regulatory connections and crosstalk processes that potentiate more than one signaling pathway. These components could become essential targets in making plants more resistant to different stresses. Coupled with the intracellular signaling systems, the recent identification of systemically propagating $Ca^{2+}$ and ROS waves in plants has unraveled a new and exciting cell-to-cell communication pathway that, together with electric signals, provides a working model demonstrating how plant cells transmit long-distance signals via cell-to-cell communication mechanisms (Gilroy et al. 2014). The combination of techniques such as forward and reverse genetic analysis, genomics and proteomics tools, microarray analyses, and big data are likely to yield exciting results and enhance understanding of plant signaling in the not too distant future. Much as the crosstalk between different cell signaling pathways, this interdisciplinary field will benefit from crosstalk between geneticists, physiologists, and ecologists (Cosgrove et al. 2000).

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# Chapter 11
# Plasmodesmata: A History of Conceptual Surprises


**Aart J.E. van Bel**

*Zur richtigen Deutung der dargestellten Befunde lässt sich auf Grund der gemachten Erfahrungen nur eine einzige Möglichkeit finden und diese entspricht der Auffassung, dass die Protoplasmakörper der inneren Zellen des Endosperms sich mit dünnen, in den feinen Verbindungskanälen verlaufenden Strängen unter einander in Verbindung setzen und so zu einer Einheit höherer Ordnung zusammentreten.*

The statement of Eduard Tangl (1879) about the intimate interdependence of plant cells and the symplasm concept.

**Abstract and Prologue** Since the initial postulate of plasmodesmata (PDs) and their function (Tangl 1879), three books have captured the progress in plasmodesmal (PD) research. The first (Gunning and Robards 1976) surveyed PD research extending over approximately 100 years. At the time of its publication, electron microscopy had confirmed the existence of previously putative intercellular cytoplasmic channels without a clear notion of the ultrastructure. PDs were no longer regarded as redundant evolutionary appendices, but solid evidence of their function was lacking. Over the years the hypothesis that higher plants were subdivided into symplasmic domains having some physiological role had been strengthened, but the significance of these domains remained uncertain. Exchange of low molecular weight solutes through PDs was a likely (but unproven) option, whereas passage of macromolecules was beyond the horizon of possibility at the time (Carr 1976).

Over two decades later, two other books (van Bel and van Kesteren 1999; Oparka 2005) illustrated the booming interest and progress in the preceding years. Doubts on issues put forward in the first book had been eliminated. Symplasmic domains were demonstrated to be of paramount importance for transport physiology (root transport, phloem transport) and developmental biology. The

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PD substructure had been assessed better and a number of components associated with PDs were identified. PDs turned out to be subject to strictly regulated constriction and dilation. The functional PD molecular exclusion limits were found to vary between the initially measured 1 kDa up to about 60 kDa. The latter values gave rise to the concept of macromolecular trafficking through PDs. As a result, new concepts on PD significance for short-distance and long-distance signaling emerged, including the key role of PDs in intercellular and long-distance transport of plant viruses.

This chapter is subdivided in line with the emergence of techniques that enabled new waves of experimental approaches in PD research. The discoveries that collectively led to the current concepts on PD structure and function are presented in chronological order. The emphasis is on the development of PD research rather than on the present state of the art. Therefore, recent research is discussed in less detail than work from the period before 2000.

## 11.1 The Microscopy Era (1880–1950)

As early as 1879, Eduard Tangl reported the existence of intercellular protoplasmic strands, which he called "Protoplasmafortsätze," between endosperm cells of *Strychnos nux vomica*. He interpreted microscopy images of tissue sections as proof of a protoplasmic continuity between cells that would allow rapid and concerted interaction between plant cells (Tangl 1879). Although this conclusion turned out to be correct, he probably did not observe the protoplasmic channels. Currently, we know that PDs (protoplasmic bonds) possess an optical diameter of up to 50 nm, which is far below the resolution threshold of high-quality light transmission microscopes because of the limitations imposed by the wavelength. Therefore, PDs are out of the reach of light microscopy. PDs in the onion epidermis, for instance, cannot be distinguished under a light microscope, but they can be exactly localized by aniline blue staining of the callose collars around PDs (Currier and Strugger 1956). Even when Hechtian threads that remain connected to the PD region are clearly visible in plasmolyzed cells of onion epidermis, the corresponding PDs remain indiscernible (Oparka et al. 1994).

An interesting question is what Tangl may have seen. My interpretation based on today's knowledge is that he observed the pit channels arising in thick secondary cell walls. The original pit fields that often contain several PDs are kept free when layers of secondary wall material are being deposited onto the primary wall after conclusion of cell expansion. Such pit channels frequently occur in walls between sclerenchyma cells, appearing as single or double lines that radiate from the cell center (Esau 1977; Bowes 2001; Wanner 2004) and look like the structures reported by Tangl. It is possible that pit channels between living sclerenchyma cells contain functional PDs (e.g., Kong et al. 2015). Therefore, Tangl's claim to have observed

what is now known as symplasmic continuity may have been correct. However, there could be an alternative explanation for his results.

Tangl described the intercellular threads as filled with a yellow or brown substance, colored by JKJ (Lugol's iodine); he interpreted this substance as being protoplasmic content. He further described how colored threads in damaged cells continued through cell walls into intact cells, where they were connected to "dark balls." The description "dark balls" matches dark-purple JKJ-stained starch grains, which constitute one of the storage compartments of *Strychnos* endosperm cells (Kooiman 1960). As Tangl himself admitted, the preparative chemical treatments were harsh and damaged most cells. The treatments may have dissolved the plasma membrane and degraded starch. Therefore, the yellow/brown colored substance in the threads might have been a degradation product of starch, such as dextrins trapped in the apoplasmic space of the pit channels. Hence, the observed symplasmic continuity may have been an artifact.

Irrespective of presumptive flaws in his visual interpretations, the merits of Tangl's ideas remain enormous. His ideas resulted in a revolutionary new view of plant organization that was fundamentally different from that of animals. The dominant animal research in the nineteenth century regarded cells as strictly autonomous units, at least structurally, assembled in organs (Schleiden 1838; Schwann 1839). As a side note, this dogma turned out to be not entirely true given the intercellular messaging via connexons in several animal organs (Saez et al. 2003) and the existence of tunneling nanotubes through which complete organelles move from cell-to-cell (van den Biggelaar et al. 1986; Rustom et al. 2004). More than a 100 years later, we regard plants as being composed of cell "colonies" operating in varying alliances and dependent on the endogenous and exogenous conditions. These cell clusters are demarcated by a permanent absence of PDs or by PDs that are closed permanently or temporarily. During periods of symplasmic isolation, adjacent cells or cell groups can operate independently, undisturbed by interfering messages from neighboring cells. These and similar concepts form the precious heritage of Tangl's work.

The intercellular connections were named "plasmodesmata" in an extensive and meticulous study by Strasburger (1901). This publication discloses that Strasburger correctly determined the location of PDs, but that the observation of PDs themselves was erroneous because he only described the origin and development of pit channels:

"*Unsere entwicklungsgeschichtlichen Untersuchungen führten somit zu dem Ergebnis, dass die Plasmaverbindungen nicht auf Zelltheilungsvorgänge zurückzuführen sind. Sie stellen nicht ausgesparten Vebindungsfäden der Kerne in den Membranen dar; sie werden vielmehr in letztere nach deren Anlage eingeschaltet. Das geschieht in den jüngsten Stadien der Membran, unter allen Umständen vor Beginn ihrer sekundären Verdickung. Die Bildung der Plasmaverbindungen erfolgt im allgemeinen nur an bestimmten Stellen der Membran bei der Tüpfelbildung*" (Strasburger 1901).

In short, Strasburger concluded that PDs are not formed during cell division but are inserted, starting from the formation of secondary cell wall. He argued that other scenarios were unlikely because PDs are evenly distributed over the tangential, radial, and transverse walls in many tissues. He argued that if PDs were formed during cell division, then non-division walls would be devoid of PDs (Strasburger 1901). At present, we know that primary PDs are formed in the cell plate between dividing cells (Porter and Machado 1960; Jones 1976; Hepler 1982) and that secondary PDs are inserted in non-division walls (Ehlers and Kollmann 2001). In conclusion, the description of the origin of PDs exactly matches the emergence of pit channels and seems to confirm that Strasburger and other researchers of his time reported the wall corridors for PDs because of the insufficient resolution of their light microscopes. This illustrates well how deceptive even meticulous observations can be if proper equipment is not available. Nevertheless, this lapse in optical interpretation did not invalidate the conceptual significance of these pioneering studies. By counting pits or pit channels, it was possible to locate putative PD connections, showing that PDs were sometimes unevenly distributed over their respective cell interfaces (Strasburger 1901). In keeping with Tangl's opinion (1879) (*"Analogie mit dem Baue der Siebröhren spricht sich in beiden vorhin behandelten Fällen noch deutlicher und so bestimmt aus, dass man die betreffenden Endospermgewebe geradezu als mit Siebplatten ausgestattete Parenchymgewebe ansprechen könnte"*), Strasburger advocated that PDs and sieve pores have an identical origin and postulated that sieve pores are composed of fused PDs.

An explosion of publications on PDs in the late nineteenth century pursued a number of "modern" issues. Goebel (1897) and Townsend (1897) recognized several principles that are still important in PD biology. Goebel (1897) suggested that cells without symplasmic contacts would be capable of autonomous and distinct development, one of the paradigms in present-day developmental plant biology. Townsend (1897) discovered that enucleate protoplasts connected to a nucleate protoplast were capable of cell wall formation, whereas loose enucleate protoplasts were not. This illustrates another paradigm in developmental biology: information can be passed on via PDs. PD-mediated exchange of messages and solutes was further substantiated by the chloroplast distribution in variegated plants (e.g., Correns 1909), the cell-to-cell diffusion of anthocyans (e.g., Bauer 1930), and a multitude of other studies (summarized in Carr 1976).

After 40 years of intensive research, the interest in PDs faded in the 1920s, probably due to the lack of decisive breakthroughs. Moreover, excitement regarding discovery of the plasmalemma (now called plasma membrane) suppressed the interest in PDs (Carr 1976). Despite the virtual standstill of PD research, some major conceptual progress was made during this period. Transport through PDs formed an integral part of the backbone of the mass flow concept (Münch 1930, pages 73–78). A quantum leap in PD research, not recognized at the time, was the application of fluorochromes. Intercellular movement of membrane-impermeant fluorescein between *Cucurbita pepo* hair cells disclosed symplasmic movement and polar transport via PDs (Schumacher 1936). About 50 years later, the use of fluorochromes provided a basis for unparalleled progress in PD research and

received belated, highly deserved appreciation. No further noticeable progress in PD research was made until the advent of electron microscopy.

## 11.2 Application of Electron Microscopy (From 1955 On)

Electron microscopy enabled observation at much higher resolutions and confirmed the existence of PDs, even though observation of plasma membrane was not possible in the pioneering studies (Mercer 1956). Conclusive evidence on PD continuity was provided by studies using osmium tetroxide fixation (Buvat 1957; Strugger 1957). A few years later, it was established that the origin of PDs is associated with cell-plate formation during cell division (Porter and Machado 1960; Jones 1976; Hepler 1982).

As a new component in the PD ultrastructure, a continuity of the ER via PDs was postulated after fixation with potassium permanganate, which resulted in improved preservation of membrane structures (Whaley et al. 1960). The existence of the ER corridor and, if existent, its nature were the subjects of heavy disputes. Initially, the discussion revolved around the question of whether the ER was linked with PDs (e.g., Esau 1963). After publication of irrefutable visual proof of an ER tubule inside the cytoplasmic PD sleeve (Lopez-Saez et al. 1966), the debate focused on the permeability of the cytoplasmic corridor and/or the ER. Although the permeability of the cytoplasmic sleeve was almost dogma, that of the ER tubule remained a matter of debate.

The initial impression was that the ER membrane inside the PD corridor was pressed against a massive densely packed rod (Lopez-Saez et al. 1966). By contrast, Robards (1968a, b) advocated an open configuration on the basis of transverse sections of PDs (Gunning and Robards 1976). This open desmotubule (Robards 1968a) had an outside granular texture and was traversed by a so-called central rod (Overall et al. 1982). On the basis of calculations on the spatial molecular structure, it was postulated that the desmotubule had to be an open structure (Gunning and Overall 1983). This view conflicted with the observation that a glutaraldehyde/osmium tetroxide/potassium ferricyanide mixture failed to stain the desmotubule region, whereas the contents of the other ER structures were densely stained (Hepler 1982). The discussion about the PD substructure subsided until more sophisticated and less damaging fixation methods, such as freeze-substitution, became available.

## 11.3 Introduction of Radioactive Compounds and Electrical Probing (1960–1980)

### *11.3.1 Polar Symplasmic Transport Through Plasmodesmata*

By the time the existence of PDs was established, renewed interest in the function of PDs had arisen. Novel chemical tools, such as radioactive compounds and metabolic inhibitors, facilitated investigations on the functional significance of PDs. The outstanding pioneering work of Arisz (1958, 1960, 1969), using excised leaves of the water plant *Vallisneria spiralis,* provided new insights into the physiological properties of PDs. Local excision of the parallel major veins left parenchymatous bridges through which compulsory symplasmic transport of radiolabeled substances took place (Arisz 1958, 1960). By contrast, local excision of parenchyma tissues imposed exclusive vascular translocation without any involvement of the usual driving forces (Arisz 1960). Phloem transport was virtually absent because the leaves were separated from the root system and the underdeveloped xylem system was not exposed to transpiration in submersed leaves (Arisz 1969).

In halved leaves, $^{14}$C-serine applied to the leaf tip moved toward the leaf base more rapidly than in the reverse direction (Arisz 1969). Excision of vascular windows hardly retarded longitudinal movement of ions (Arisz 1960). Simultaneous application of the metabolic inhibitor KCN strongly suppressed uptake of $^{36}$Cl, whereas application of KCN near the bridges had no effect (Arisz 1958). In tandem, these results indicated a rapid polar symplasmic PD-mediated solute transport that is independent of metabolism. This apparent controversy between polarity and metabolic independence did not emerge in another transport study, in which longitudinal symplasmic transport of $^{14}$C-serine or $^{14}$C-alanine in linear leaves of *Sagittaria graminea* was strongly reduced under darkness (Schenk 1972). Thus, the controversy over metabolic involvement in PD gating remained unresolved at the time.

### *11.3.2 Electrical Conductance of Plasmodesmata*

Intracellular impalement of microelectrode tips provided information on the electrical conductivity of PDs as a measure of their functionality. In a first approach, a current pulse was injected via an injector electrode into one of the linearly arranged cells of *Nitella translucens*. The strength of a passing pulse was recorded by a receptor electrode several cells away from the injected cell (Spanswick and Costerton 1967). From the recorded values, electrical resistance over the cell array was calculated to be 350 times lower than the resistance over the plasma

membrane of the injected cell. This high electrical conductance of the intervening cell walls was ascribed to the presence of PDs.

The conclusion that electrical potential waves were able to propagate via PDs was supported by ready transfer of light-induced depolarizations from the green to the white regions in variegated *Oenothera* leaves (Brinckmann and Lüttge 1974). The profile and size of the potential waves in the illuminated and distant cells were similar, which demonstrated that the triggered signals in the green parts of the leaves were perceived in the white parts because of the presence of PDs.

Final evidence on the relationship between PD conductance and propagation of potential waves was provided by a linear correlation between electrical conductance and the number of PDs in *Azolla* root tips. These roots grow by successive divisions of one tip cell that continuously produces spirally positioned merophyte daughter cells until a determinate root length has been reached (Gunning 1978). Electrical coupling between the tip cell and its daughter cells declines with growth of the root (Overall et al. 1982). This phenomenon was attributed to the fact that the number of PDs decreased with every cell division (Gunning 1978). As a long-forgotten observation, comparable to the phenomena in *Cucurbita* trichomes (Schumacher 1936) and *Vallisneria* leaves (Arisz 1960), PDs in *Azolla* exhibited polar behavior: the PD conductance in the apical direction exceeded that in the basal direction (Overall et al. 1982).

## 11.4 Use of Fluorochromes (From 1980 On)

### *11.4.1 Molecular Exclusion Diameter of Plasmodesmata*

By the end of the 1970s, fluorescence microscopy (Schumacher 1936) was rediscovered as a tool for PD research (Goodwin 1976). Membrane-impermeant fluorochromes were injected into cells via microcapillaries with tip diameters of approximately 1 μm. Charged fluorochromes could be driven into the cell by iontophoresis or pressure. In later years, uncharged high molecular weight fluorescent compounds were injected using pressure devices. Later, a multipurpose device was developed in which iontophoresis, pressure injection, and membrane potential measurements were integrated. This tool could be used for each of the functions or for simultaneous injection of two dyes with different emission spectra (Kempers et al. 1999).

The essence of this approach is that, once a membrane-impermeant fluorochrome is injected, it can only escape to other cells via the PDs. This technique was initially explored in *Elodea canadensis* (later named *Egeria densa*) leaves (Erwee and Goodwin 1983), but injection into plant hairs, with linearly aligned cells, facilitated more discrete assessment of PD properties (Tucker 1982; Tucker et al. 1989; Terry and Robards 1987). In a series of fascinating, exploratory studies, Goodwin and Erwee (Goodwin 1983; Erwee and Goodwin 1983, 1984; Goodwin et al. 1990) demonstrated the feasibility of the approach. They used carboxyfluorescein linked

with amino acid tails of various lengths to investigate PD permeability to solutes of different molecular weight and chemical nature (Erwee and Goodwin 1983). The cut-off molecular size for intercellular transport (size exclusion limit or molecular exclusion diameter) between *Elodea* leaf cells appeared to be in the order of 1 kDa. (Goodwin 1983). Aromatic amino acids moved more slowly than aliphatic ones (Erwee and Goodwin 1984). Plasmolysis disrupted PD continuity. After deplasmolysis, however, PD connectivity was restored and the size exclusion limit of PDs became larger (Erwee and Goodwin 1984). The spread of carboxyfluorescein was appreciably reduced by addition of $Ca^{2+}$, which suggested that PDs were gated by means of a $Ca^{2+}$-regulated mechanism (Erwee and Goodwin 1983). Pitfalls in the use of fluorescent dyes are the ready sequestration of fluorochromes in membrane-lined cell compartments, mainly the vacuole (Tucker et al. 1989), and the putative impact of microcapillary penetration on PD gating (Radford et al. 1998; van Bel and Ehlers 2005). Both strongly limit natural PD-mediated fluorochrome dispersion.

Similar experiments using *Setcreasea* staminal hairs (Tucker 1982; Tucker et al. 1989) and *Abutilon* nectary trichomes (Terry and Robards 1987) confirmed the results and conclusions obtained with *Egeria*. Molecular mobility was found to be dependent on the hydrodynamic radius of amino acids (Terry and Robards 1987) and their diffusion coefficients (Tucker et al. 1989), with the restriction that amino acid diffusion through PDs was also dependent on the relative charge (Tucker and Tucker 1993); the latter had been implicitly shown earlier in *Egeria* leaves (Erwee and Goodwin 1983). Furthermore, vacuolar sequestration reduced symplasmic transport of carboxyfluorescein and its derivatives in *Setcreasea* staminal hairs (Tucker et al. 1989). Later studies showed that this fluorochrome sequestration and additional compartmentation were complex processes (Wright and Oparka 1994).

### *11.4.2 Permanent Symplasmic Domains Caused by Absence of Plasmodesmata*

During the 1980s, it became obvious that functional PDs were a prerequisite for diffusional transport, rapid intercellular communication, and electrical connectivity and signaling. On the basis of electron microscopy images, the frequencies of PDs at various interfaces were counted and charted in so-called plasmodesmograms (coined by van Bel et al. 1988). Even though their usefulness is limited for the prediction of intercellular transport capacities (van Bel and Oparka 1995), plasmodesmograms reveal cellular interfaces where PDs are entirely or virtually lacking. At either side of these interfaces, autonomously acting symplasmic cell domains operate. Chemical communication between such domains is only possible by transfer of molecules over two plasma membrane barriers, and is thus selective and relatively slow.

The borderline between two permanently separated symplasmic domains forms a drastic intermission in symplasmic continuity, with clear purposes. For instance, direct contact between tissues with a different genome needs to be avoided, because otherwise conflicting genetic directives arise. The borderline between maternal and embryonic tissues in seeds is therefore devoid of PDs (Patrick and Offler 1995, 2001). As an example of functional symplasmic isolation in mature plants, absence of PDs between guard cells and subsidiary cells in stomata (Wille and Lucas 1984) enables their different reactions to the same changing light conditions, which makes the cellular interplay in the stomatal apparatus so efficient. During stomatal maturation, PDs first become dysfunctional (Palevitz and Hepler 1985), as shown by microinjection of Lucifer Yellow, and are then removed (Wille and Lucas 1984). Although the guard cells in *Allium* (Wille and Lucas 1984; Palevitz and Hepler 1985) and *Commelina* (Erwee et al. 1985; Palevitz and Hepler 1985) are fully symplasmically uncoupled from each other, the PDs remain interconnected between the dumbbell-shaped guard cells of grasses (Srivastava and Singh 1972; Mumm et al. 2011).

As another example of permanent symplasmic discontinuity, PDs between the mesophyll symplasm and the sieve element–companion cell complexes are virtually absent in minor veins of apoplasmically phloem-loading species (Gamalei 1989). Near-isolation of the symplasmic domains here enables accumulation of photosynthate in the sieve elements (Geiger et al. 1973; Giaquinta 1983), giving rise to pressure-driven mass flow (Münch 1930). The discovery of a symplasmic continuity between the mesophyll symplasm and sieve element–companion cell complexes in other species (Fisher 1986; Gamalei 1989) seemingly conflicted with photosynthate accumulation and setup of mass flow, until the photosynthate trapping mechanism was proposed (Turgeon 1991).

### *11.4.3 Symplasmic Domains Caused by Constriction of Plasmodesmata*

Symplasmic domains are less discrete when PDs are present along the borderline, but are permanently or temporarily closed. Such domains can be identified by fluorochrome injection or by measurement of membrane potentials. Use of Lucifer Yellow injected into sieve elements of *Ricinus communis* and *Salix alba* disclosed that the sieve element–companion cell complexes in transport phloem are symplasmic domains that become virtually disconnected from adjacent cells under source-limiting conditions (van Bel and Kempers 1990; Patrick and Offler 1996).

Membrane potential mapping demonstrated that nonvascular tissues are also subdivided in symplasmic domains (van der Schoot and van Bel 1990). It is to be expected that cells have approximately identical membrane potentials if they are symplasmically coupled. If adjacent cells possess disparate potentials, symplasmic

discontinuity between them is likely. The identification of clusters of cells in tomato stems, with identical membrane potentials within each cluster, hinted at the existence of symplasmic domains; this was corroborated and further supported by the potential pattern that coincided with the dye coupling (van der Schoot and van Bel 1990).

That PDs are closed at certain borderlines can only be made visible using fluorochromes. Unexpectedly, fluorochromes cannot cross the borderline between basal trichome cells and mesophyll in *Abutilon* leaves, in spite of numerous PD contacts (Terry and Robards 1987). The isolation renders trichomes symplasmic domains, but PD closure at this interface does not need to be permanent. Single-celled cotton fibers are transiently symplasmically isolated from neighboring cells to enable rapid cell elongation (Ruan et al. 2001). These experiments demonstrate that PDs can be closed reversibly for many hours in response to developmental or physiological demands.

### *11.4.4 Constriction of Plasmodesmal Pathways*

The reversible opening and closure of PDs raised questions regarding regulation. The negative effects of $Ca^{2+}$ ions on PD permeability (Erwee and Goodwin 1983) indicate a relationship between presumptive collar-like callose depositions around the PD orifices and constriction of the PD pathway. At the time, evidence was available for a $Ca^{2+}$ dependence of callose synthesis (Kauss 1987); therefore, a relationship between $Ca^{2+}$ levels, callose synthesis, and PD constriction was obvious.

PDs were reported to be constricted in the PD neck regions (Robards 1976; Gunning and Robards 1976). Similar neck constrictions occurred in PDs of leaf cells of *Salsola kali* and between root cells of *Epilobium hirsutum* fixed with tannic acid (Olesen 1979) and, with some degree of reserve, in freeze-substituted salt glands of *Tamarix* (Thomson and Platt-Aloia 1985). Although callose collars were constitutively integrated in PD models (Robards and Lucas 1990), some doubts remained about the universality of callose-based neck constrictions (Lopez-Saez et al. 1966; Gunning and Overall 1983). Neck constrictions appeared to be absent, for instance, between gametophyte cells of *Onoclea sensibilis* (Tilney et al. 1991) and root-tip cells of *Zea mays* (Turner et al. 1994), even when using more preservative fixation procedures. However, the occurrence of callose deposition around PD orifices between many cell types is beyond doubt because aniline blue-stained callose aggregations were also well recognizable under the light microscope in living tissues (e.g., Radford et al. 1998; Ruan et al. 2004; Sagi et al. 2005; Levy et al. 2007; Faulkner et al. 2013).

In conclusion, by the end of the1980s, consensus existed that PDs were involved in intercellular transport, had a universal size exclusion limit of about 1 kDa, and were reversibly gateable, probably under the influence of $Ca^{2+}$. Due to findings based on fluorochrome injection, new concepts emerged regarding the role of PDs

in plant organization. The absence of PDs renders symplasmic domains permanently separated, whereas the presence of PDs does not necessarily imply symplasmic connectivity. The most intriguing notion for further research was $Ca^{2+}$-regulated reversibility of PD closure, which would enable a dynamic management of symplasmic domains under the control of cytoplasmic $Ca^{2+}$.

## 11.5 High-Resolution Electron Microscopy (From 1990 On)

### *11.5.1 Plasmodesmal Ultrastructure*

New technical developments were responsible for the next generation of observations on PD ultrastructure. Freeze-fractioning and freeze-substitution had less impact on the fine structure of PDs than chemical fixation (Thomson and Platt-Aloia 1985; Ding et al. 1992b; Tilney et al. 1991; Turner et al. 1994). This preservative approach expanded knowledge about the PD substructure to an appreciable extent. In cell-division walls, both plasma membrane and ER membrane within the PD corridors were shown to be covered with macromolecular complexes that were tightly pressed together or intimately linked (Ding et al. 1992b). With maturity, the space between the globular complexes on either membrane side widened, giving rise to a central cavity that was bridged by filamentous molecules (spokes), connecting both types of globular bodies at either side of the gap (Ding et al. 1992b). The PD orifices were probably constricted by callose deposition at the mature stage (Ding et al. 1992b; Lucas et al. 1993).

Computer-enhanced digital image analysis of PDs at the Kranz mesophyll–bundle sheath interface of *Themeda triandra* not only corroborated previous work (Ding et al. 1992a, b), but also revealed a spiral arrangement of globular complexes on the desmotubular surface (Botha et al. 1993; Badelt et al. 1994). This spiral structure, revealed by a similar computer-aided approach, was ascribed to the arrangement of motor proteins inside the cytoplasmic sleeve (Overall and Blackman 1996). Silver-stained one-dimensional SDS-PAGE strips and western blots demonstrated that PD isolates contained actin, which was confirmed by the location of anti-actin antibodies in freeze-substituted electron microscopy sections of *Hordeum vulgare* and *Nephrolepsis exaltata* tissues embedded in LR resin (White et al. 1994). Moreover, the actin perturbant cytochalasin B stimulated the opening of PDs in *Nephrolepsis*. This effect indicated that PD closure is mediated by actin, which, in turn, is suggestive of energy input (White et al. 1994). Similar PD widening in the presence of cytochalasin D in mesophyll cells of *Nicotiana tabacum* suggested engagement of actin in control of PD permeability (Ding et al. 1996). Actin molecules were proposed to be spiraling around the desmotubule (Overall and Blackman 1996), whereas the molecular spokes (Ding et al. 1992b) were proposed to be composed of myosin (Radford and White 1998).

### 11.5.2 Membrane Continuity Between Cells: Pasmodesmal Corridors

Until now, little attention has been paid to the intercellular continuity of the ER and plasma membrane and to the mobility of membrane components between cells via PDs. In contrast to the free traffic of cytosolic compounds up to 1 kDa, plasma membrane components do not exchange via PDs (Grabski et al. 1993), which is indicative of a lateral diffusion barrier at the cellular boundary. By contrast, ER membrane components, lipid compounds in particular, can move from cell to cell via PDs (Grabski et al. 1993). However, the availability of the desmotubule as a solute passageway between ER stacks in adjacent cells has remained a matter of debate. Space calculations predicted that only single water molecules can pass the desmotubular lumen (Overall et al. 1982). In contrast, other studies gave clues for much wider ER corridors. Lucifer Yellow, for instance, moved through a complex network of vacuolar vesicles in trichomes of *Cicer arietinum* (Lazzaro and Thomson 1996). Given the distinct fluorescent bands inside the cell walls (Lazzaro and Thomson 1996) and the continuity between vacuolar systems and ER, the ends of the vesicular system at either side of the cell walls seem to be in contact via desmotubules. Furthermore, the ER including the desmotubules was also regarded as a potential part of the phloem-loading pathway, given the numerous vesicles in companion cells of symplasmically loading species (Gamalei et al. 1994). Such a desmotubular phloem-loading pathway may indeed exist in gymnosperms, where PDs are traversed by ER (Kollmann and Schumacher 1963; Glockmann and Kollmann 1996; Schulz 1992).

### 11.5.3 Plasmodesmal Development and Neck Constrictions

Transmission electron microscopy images (Ding et al. 1992b) confirmed claims on the occurrence of PDs without and with neck constrictions (or sphincters) imposed by callose collars (see Sect. 11.4.4). Young PDs possess tightly appressed ER membranes and plasma membranes, whereas mature PDs seem to have much wider central cavities and neck constrictions. The question then shifted to whether there is universal occurrence of neck constrictions in mature PDs. Using glutaraldehyde fixation or freeze-substitution, neck constrictions were reported, as in previous work (see Sect. 11.4.4), for PDs in the ferns *Nephrolepsis* and *Azolla*, a water plant (*Spirodela*), and *Hordeum* (Badelt et al. 1994), suggesting that callose-surrounded sphincters are ubiquitous in the plant kingdom. Nevertheless, doubts remained about the presence of sphincters in intact tissue. Callose deposition was ascribed to turgor shocks and wounding during tissue fixation or microelectrode impalement prior to injection (Radford et al. 1998). However, there are sufficient reasons to believe (see Sect. 11.8.2.1) that neck constrictions exist naturally, albeit not in all PDs. Comparative images of *Nicotiana clevelandii* PDs showed a

difference between the neck regions of mesophyll PDs (no constrictions) and hair cell PDs (probably neck constrictions) fixed under identical conditions (Waigmann et al. 1997).

### *11.5.4 Primary and Secondary Plasmodesmata: Semantic Discourse*

The confusing use of the terms "primary" and "secondary" PDs (e.g., Ding et al. 1992a, 1993; Volk et al. 1996) caused a veritable tower of Babel among PD researchers in the mid-1990s. Single-stranded PDs had been qualified as primary, and branched PDs as secondary, irrespective of their origin (Lucas et al. 1993). This confusion came to an end thanks to two authoritative reviews on this issue (Kollmann and Glockmann 1999; Ehlers and Kollmann 2001). Kollmann and Glockmann (1999) stated that branching of PDs is not related to their origin (primary or secondary PDs). They concluded that secondary PDs occur at non-division walls and fusion walls originating from different meristems or organisms. Some examples are found in carpel fusion walls; between cells in tyloses, cell cultures, graft unions, and chimeras; and between hosts and their parasites (Kollman and Glockmann 1999). After a series of studies on diverse objects (Ehlers and Kollmann 1996; Ehlers et al. 1996; Glockmann and Kollmann 1996), Ehlers and Kollmann (2001) pointed out once again that the only meaningful criterion for the use of "primary" or "secondary" was the stage of cell development at which PDs were being formed. PDs formed in the cell plate had to be named "primary," even when they start branching or fusing during secondary cell-wall apposition (Ehlers and Kollmann 2001). By contrast, PDs that emerged de novo in existing cell walls had to be termed "secondary" (Ehlers and Kollmann 2001). According to this definition, single-stranded PDs could well be secondary. These definitions proved to be a useful basis for a generally accepted nomenclature.

### *11.5.5 Formation of Primary and Secondary Plasmodesmata*

As demonstrated by early research (Porter and Machado 1960; Jones 1976; Hepler 1982), primary PDs are established by ER entrapment in the growing cell plate (Kollmann and Glockmann 1999). At later stages of cell development, ER branching can give rise to branching of primary PDs or even coalescence of PDs in division walls (Ehlers and Kollmann 2001). These events result in a wide variety of PD structures (Kollmann and Glockmann 1999): single-stranded PDs (Glockmann and Kollmann 1996), H-shaped PDs (Glockmann and Kollmann 1996), complex PDs (Gamalei 1989; Volk et al. 1996), and radiating fields of

PDs (Glockmann and Kollmann 1996). All these PD structures may occur in the same plant species along with several types of secondary PDs.

Secondary PDs are formed in existing cell walls after ER strands are deployed along and anchored to the plasma membrane (see also Sect. 11.5.5) by means of minute clamps (Kollmann and Glockmann 1999) at opposite sides of the cell wall (Ehlers and van Bel 2010). ER then fuses with the plasma membrane, which is followed by local dissolution of the wall (Kollmann and Glockmann 1991). The first attempts to understand the mechanism of cell wall dissolution hint at the involvement of peroxidases in cell wall degradation (Ehlers and van Bel 2010). Superoxide and $H_2O_2$ are produced locally by the ER and generate radicals (hydroxyl ions, $^{\cdot}OH$) with an ultrashort life that act as cell wall loosening agents with a limited action radius (Ehlers and van Bel 2010).

Until the discovery of longitudinal PD fission (Ehlers et al. 2004; Faulkner et al. 2008; Ehlers and van Bel 2010), loosely paired simple PDs and H-shaped PDs had been interpreted as transitional stages in the formation of branched PDs by lateral fusion (Glockmann and Kollmann 1996; Itaya et al. 1998; Oparka et al. 1999; Roberts et al. 2001). The striking occurrence of PD twins (Ehlers et al. 2004), however, led to the notion that PD multiplication in mature walls is not only caused by insertion of single secondary PDs, but also by longitudinal division of existing PDs that serve as templates for the insertion of secondary PDs (Faulkner et al. 2008).

### *11.5.6 Formation of Secondary Plasmodesmata in Graft Unions*

The formation of secondary PDs was studied extensively in graft unions (Kollmann et al. 1985; Kollmann and Glockmann 1991). The formation and linking of secondary PDs in adjacent walls of stock and scion was identified as an absolute prerequisite for the success of grafts. Crucial for PD coupling is the precise opposite ER positioning at either side of the wall, which must be finely orchestrated (Kollmann et al. 1985). Any secondary PD constructed without a counterpart at the opposite side is aborted (Kollmann et al. 1985); ubiquitin is engaged in the selective degradation of PDs (Ehlers et al. 1996). By exception, grafting between species of different plant families is successful (Kollmann and Glockmann 1985). Usually, intraspecific grafting leads to PD linking, and only between the vascular areas of stock and scion (Kollmann et al. 1985). As detailed in Sect. 11.9.5, grafts are excellent tools for study of the systemic transfer of proteins and genetic information (Golecki et al. 1998; Kim et al. 2001; Turnbull et al. 2002; Paultre et al. 2016).

### *11.5.7 Diversity of Plasmodesmata Configurations*

Several studies have revealed a morphological variety of PDs (see Sect. 11.5.4) in one and the same plant, organ, or tissue (e.g., Robinson-Beers and Evert 1991, in *Zea* leaves; Glockmann and Kollmann 1996, in *Metasequoia*; Volk et al. 1996, in cucurbit leaves; Waigmann et al. 1997, in *Nicotiana* leaves; Botha et al. 2005, in grass leaves; and Ehlers and van Bel 2010, in tomato cambium). It seems unlikely that morphologically distinct PDs at different interfaces exert identical functions.

The most informative cells to study for clarification of this issue are sieve element–companion cell complexes, which originate from the same mother cell and have identical PDs at each interface. After a longitudinal division, three types of PDs develop at the different interfaces of the complex: sieve pores to the next sieve element at the transverse wall, pore–PD units (PPUs) between sieve elements and companion cells, and single-stranded PDs between companion cells and phloem parenchyma cells (van Bel 2003). The latter PDs have a much lower electrical conductivity than PPUs and sieve pores (van Bel and van Rijen 1994) and constitute a symplasmic bottleneck in the transport phloem (Kempers et al. 1998); thus, their collective transport capacity is much lower than that of PPUs (van Bel 2003). Moreover, their molecular machinery may deviate from other PDs, as suggested by the finding that they are unable to traffic phloem-limited poleroviruses (Peter et al. 2009) and luteoviruses (Brault et al. 2011), which is necessary for phloem containment. If such a PD functioning is a general phenomenon, the quest for a universal molecular structure of PDs may be senseless, because diversity and functional specialization are logically associated with variations in molecular structure.

## 11.6 Introduction of Protein Separation Techniques (From 1985 On)

### *11.6.1 Sieve Element Proteins and Their Putative Consequences for Plasmodesmal Transfer to Sieve Elements*

Protein separation techniques, such as SDS-PAGE, allowed visualization of the protein composition in sieve-tube exudate, which presumably reflects the *in vivo* content of sieve element lumina. Given the turnover of sieve element proteins (Fisher et al. 1992), permanent PPU-mediated transfer of proteins produced in the nucleate companion cells to the enucleate sieve elements seems imperative. The plethora of proteins in the sieve-tube sap (Sakuth et al. 1993; Schobert et al. 1995) suggests that PPUs are capable of transporting a broad spectrum of proteins that are variable in size, structure, and charge distribution.

### *11.6.2 Substructure and Macromolecular Building Blocks of the Plasmodesmal Apparatus*

Until 1990, protein separation techniques had mainly been used in plant science for identification of sieve element proteins (Cronshaw and Sabnis 1990) and were amply employed for the same purpose in later years (see Sects. 11.6.1 and 11.7.3). With an increase in technical possibilities, identification of the PD proteome became a focus area in PD research. As a first attempt, PD proteins from the cell wall fraction of *Solanum nigrum* epidermis homogenates were isolated and separated; two proteins of 28 kDa and 43 kDa were identified (Monzer and Kloth 1991). Using a similar approach, two PD proteins, PAP 26 and PAP 27, were detected; PAP27 that was localized at the PD orifices (roughly the neck regions) cross-reacted with an antibody against region 100–122 of rat heart connexin 43 (Yahalom et al. 1991). It was *en vogue* at the time to search for structural similarities between connexins and PD proteins (Meiners and Schindler 1989; Meiners et al. 1991). An improved isolation procedure for the isolation of PDs yielded eight presumptive PD-bound proteins (Kotlizky et al. 1992). Remarkably, PAP27 was absent among the proteins detected in this approach (Kotlizky et al. 1992) and was claimed to be a protein kinase on the basis of its sequence (Mushegian and Koonin 1993).

There followed a period of numerous attempts to isolate PD-associated proteins (e.g., Epel et al. 1995); a fixed set of proteins was expected, but the sets of proteins detected were highly variable. It was unclear whether this diversity was caused by fundamental differences between the molecular machineries of the respective PDs (see Sect. 11.5.6) or to inadequate separation and isolation. PD proteins were collected from cell wall isolates. During cell disruption, variable amounts of plasma membranes and ER membranes remained attach to PDs enclosed in the cell wall. Technical limitations frustrated the progress of proteome research for a considerable number of years.

## 11.7 Introduction of Fluorochrome-Conjugated Dextrans and the Use of Transgenic Plants (From 1990 On)

### *11.7.1 Plasmodesmal Permeability and Regulatory Factors*

Originally, the opening of PDs was deemed to be an active state requiring a supply of energy. However, counterintuitively, the opposite was found to be true: PD closure appears to be energized, whereas PD dilation (widening) is associated with an interference in the energy supply. The positive effect of cytochalasin B on PD widening (White et al. 1994) was compatible with the positive PD permeability responses to the metabolic inhibitor sodium azide in *Setcreasea* staminal hairs (Tucker 1993) and *Hordeum* root cells (Cleland et al. 1994). Azide doubled

the molecular exclusion limit of the PD staminal hairs (Tucker 1993) and increased the exclusion limit by a factor 5–7 in the roots (Cleland et al. 1994). Other metabolic inhibitors, such as CCCP and probenecid, promoted symplasmic movement from the sieve elements to the root cortex cells (Wright and Oparka 1997). The data suggested that some form of energization was engaged in PD closure. In contrast, anaerobic conditions did not alter the electrical resistance between cortex cells, which was taken as evidence for the absence of metabolic involvement in PD opening (Zhang and Tyerman 1997).

More insight was gained into the underlying mechanisms of PD constriction when the negative effects of $Ca^{2+}$ on PD permeability (Erwee and Goodwin 1983) were also found for PDs in soybean root cells (Baron-Epel et al. 1988) and *Setcreasea* staminal hairs (Tucker 1990). Strikingly, inositol 1,4 bisphosphate and to a lesser degree 1,4,5 trisphosphate ($IP_3$) inhibited symplasmic cell-to-cell transfer of carboxyfluorescein, whereas other inositol derivatives did not (Tucker 1988). PD constriction was attributed to an indirect $Ca^{2+}$ effect, because $IP_3$ had been found to stimulate $Ca^{2+}$ efflux from ER cisternae. The hypothesis was tested by injection of the toxic peptide mastoparan from wasp venom (Tucker and Boss 1996), which induces the availability of G-protein and, hence, stimulates cytosolic $IP_3$ synthesis and increases $Ca^{2+}$ release from the ER (Todokoro et al. 2006). In addition to $Ca^{2+}$, cell turgor was found to be involved in the regulation of PD permeability, although it was also suggested that PD conductivity (as measured by the passage of electrical currents) is regulated by osmo-sensing rather than turgor sensing (Lew 1996). Ingenious clamping experiments to measure the turgor pressure in individual cells of uniseriate hairs demonstrated that pressure differences of more than 200 kPa triggered PD closure between contiguous cells (Oparka and Prior 1992). This indicated the existence of a PD-located pressure valve between the cells, in agreement with the previously reported polar transport (Schumacher 1936; Arisz 1969; Schenk 1972; Overall et al. 1982). It was unclear whether the sensor resides inside the cytoplasmic sleeve or is a result of turgor impact on regulation of the neck constriction. Phloem unloading experiments hinted at the latter mechanism (Schulz 1995). Bathing *Pisum sativum* root tips in a solution of 350 mM mannitol made the orifices of PD expand by a factor of 2.5 after 1 h, while the neck constrictions disappeared (Schulz 1995). The diameter of the central cavity was much less affected than that of the orifices, the enlargement of which was directly proportional to the (symplasmic) unloading of $^{14}C$-sucrose (Schulz 1995).

Based on these three pieces of evidence, it was accepted during the mid-1990s that both $Ca^{2+}$-dependent (probably callose deposition) and actin-associated and energized mechanisms were involved in gating of PDs (see for a definition of gating, see Schulz 1999), without much understanding of the underlying mechanisms.

### 11.7.2 Emergence of Plasmodesmata Molecular Exclusion Limits Larger than 1 kDa

Along with increased knowledge about PD closure, evidence emerged that the basal molecular exclusion limit of some PD species was larger than the 1 kDa limit measured thus far. As a new tool of research, fluorochrome-tagged uncharged dextran chains of variable size were employed to test the functional diameter at diverse cell boundaries. Initially, intercellular transport of 20 kDa FITC-dextrans in *Nitella* (Kikuyama et al. 1992) was used as an argument for larger functional diameters in higher plants, with reference to the special structure of PDs (absence of a microtubule) in Chlorophyta. The latter argument did not hold for the desmotubule-containing PDs between sieve elements and companion cells in the extrafascicular phloem of *Cucurbita maxima* (Kempers et al. 1993) and those in the fascicular phloem of *Vicia faba* (Kempers and van Bel 1997). These PD connections allowed transfer of at least 3 kDa (Kempers et al. 1993) or 10 kDa dextrans (Kempers and van Bel 1997). Conclusive proof of the large molecular exclusion limit of the unilaterally branched PDs between sieve elements and companion cells (PPUs; van Bel 1993) was given by the symplasmic transfer of the 30 kDa green fluorescent protein, manufactured in companion cells, toward sieve elements (Imlau et al. 1999).

### 11.7.3 Macromolecular Trafficking from Companion Cell to Sieve Element Through Plasmodesmata

The search for larger exclusion limits coincided with the appearance of a groundbreaking, visionary Tansley review (Lucas et al. 1993), predicting a key role for PDs in macromolecular transport. Symplasmic transfer of macromolecules would be of major importance for developmental biology (Lucas et al. 1993). Concomitantly, PD research branched off in diverse directions and boomed, not least because of the availability of several novel molecular techniques.

The most logical site for study of macromolecular transport of PDs is the cell interface between sieve elements and companion cells, where PPUs reside. The obvious reason for this choice is the wealth of proteins in the sap of the enucleate sieve elements (Sakuth et al. 1993; Schobert et al. 1995). It had been suspected that the protein production needed to sustain sieve element protein turnover (Fisher et al. 1992; Nakamura et al. 1993) relies on the nucleate companion cells (Bostwick et al. 1992). Macromolecular transport to sieve elements must be mediated by PPUs (Imlau et al. 1999) because sieve elements are symplasmically isolated from all vascular cell types other than companion cells (Kempers et al. 1998). The presence of structural proteins in both cell types was viewed as a compelling argument for protein trafficking through PPUs, as the corresponding mRNA only occurred in companion cells (Bostwick et al. 1992). Further work demonstrated that the SUT1

sucrose transporter residing in at the sieve element plasma membrane was synthesized in the companion cells (Kühn et al. 1997). At the same time, trafficking of *SUT1* mRNA to sieve elements was likely because it was targeted to PPUs (Kühn et al. 1997), with the SUT1-facilitated transfer of its own mRNA as a potential implication.

Cell-to-cell movement of sieve element proteins from *Cucurbita maxima* and *Ricinus communis* injected into mesophyll cells of tobacco (Balachandran et al. 1997) was a persuasive argument in favor of the special ability of sieve element-borne proteins to widen PDs. This raised the question of whether several sieve element proteins have the permanent ability to gate PDs, which might explain the sizeable molecular exclusion limits of PPUs (Kempers and van Bel 1997; Imlau et al. 1999) and the PPU-mediated trafficking of mRNA (Kühn et al. 1997).

### *11.7.4 Viral Transport Through Plasmodesmata*

Viral transfer between cells had already been related to PD transfer for a considerable time (Esau et al. 1967) when it became clear that viral movement proteins (MPs) carry the viral genome from cell to cell via PDs (Deom et al. 1987). Therefore, the functional diameter of PDs between mesophyll cells was tested in transgenic tobacco plants expressing the 30 kDa MP of tobacco mosaic virus. In transgenic plants, the functional diameter of PDs between mesophyll cells was upregulated to 10 kDa, as demonstrated using fluorescently labeled dextrans, whereas PDs in uninfected plants had a molecular exclusion limit of about 1 kDa (Wolf et al. 1989).

The claim that the molecular exclusion limit was affected by MPs could not be substantiated in *Nicotiana* plants carrying a variety of viruses, inferring that the stimulating MP effect on PD dilation was not permanent in wild-type plants (Derrick et al. 1990). However, PD permeability was increased during cell-to-cell spread of microinjected tobacco rattle virus (Derrick et al. 1992). The apparent discrepancy was explained by the fundamentally different virus-related PD behavior in constitutive MP transformants and wild-type plants. Although PDs in the transformants were permanently enlarged (Wolf et al. 1989), PD gating only occurred at the leading edge of the extending viral infection in wild-type plants (Oparka et al. 1997). At the forefront of the infection, nearly all PDs were targeted with GFP-labeled MPs of tobacco mosaic virus. A tenfold increase in the molecular exclusion limit was restricted to PDs at the infection front. Behind the front, the exclusion limits returned to the original values even though MPs remained in the PDs (Oparka et al. 1997).

Binding to MPs is absolutely essential for macromolecular transfer through PDs: the reporter protein GUS (beta-glucuronidase) could only move as a complex with the MP of tobacco mosaic virus (Waigmann and Zambryski 1995). GUS alone did not move from cell to cell, not even in the detached presence of the MP. Furthermore, PD gating by viral MPs was not entirely related to plant species:

the 3 kDa MP of cucumber mosaic virus (CMV) induced PD gating in transgenic tobacco plants (Vaquero et al. 1994).

As a result of these studies, the idea gained ground that MPs enhance the PD diameter along with cell-to-cell transfer of viral RNA. The fluorescently labeled 35 kDa MP of red clover necrotic mosaic virus (RCNMV) moved rapidly from cell to cell while facilitating intercellular trafficking of RCNMV RNA (Fujiwara et al. 1993). During this PD transfer, viral RNA adopted a filamentous unfolded conformation (Citovsky et al. 1992). In conclusion, viral RNA was assumed to unfold after binding to MP and to be carried through PDs as an MP–nucleic acid complex (McLean et al. 1993; Mezitt and Lucas 1996). A similar mode of PD trafficking from companion cell to sieve element was postulated for the 3a MP of CMV (Blackman et al. 1998). An alternative model of macromolecular passage was that MPs, after opening up the PD corridor and unfolding the RNA, remain at the PD orifice and linear RNA moves freely cell to cell (McLean et al. 1993).

Importantly, tobacco mosaic virus MP was found to be associated with the cytoskeleton (McLean et al. 1995). The possibility was raised that cytoskeletal actin filaments are connected to actin coils around the desmotubule (Botha et al. 1993; Badelt et al. 1994; White et al. 1994; Overall and Blackman 1996) and act as ATP-motorized conveyer belts for viral RNA transfer (McLean et al. 1995). This concept of intracellular viral transfer was corroborated by images showing that the GFP-labeled MP of tobacco mosaic virus colocalized with ER structures, denominated as viral factories, from which MPs were distributed along microtubules throughout the cell and targeted to PDs via actin filaments (Heinlein et al. 1998).

An important observation was that tobacco mosaic viruses only colocalized with "mature" PDs (Ding et al. 1992a). Such PDs are primary PDs (see Sect. 11.5.3) that branch in the secondary wall layer during the course of cell development. GFP-labeled CMV 3a MP only targeted PDs in the mature state, through which it was trafficked from cell to cell (Itaya et al. 1998). This behavior seemingly demonstrates that the macromolecular machinery of PDs during meristematic and primary cell stages is not yet fully completed or otherwise differs from that at the mature cell stage.

As a last note, a second mode of MP engagement in virus trafficking via PDs was discovered, but is less relevant for the present overview. It was demonstrated that the comovirus MP is involved in drilling tunnels through irreversibly damaged PDs to enable passage of complete virions (van Lent et al. 1990).

### *11.7.5 Macromolecular Transport Through Plasmodesmata and Its Developmental Impact*

Because the enlarging effect of MPs on the functional PD diameter (Wolf et al. 1989; Derrick et al. 1992) coincides with the trafficking of viral RNA (Citovsky

et al. 1992; Fujiwara et al. 1993), a similar mode of transfer was envisaged for trafficking of innate macromolecules to exchange positional information (Lucas et al. 1993). The idea that position rather than lineage is decisive for cell differentiation and that positional information is communicated via PDs referred to early concepts in PD research (Townsend 1897; Carr 1976).

The first evidence in favor of PD trafficking of plant macromolecules was obtained from studies of the homeobox protein KNOTTED 1 (KN1). The transcription factor *KN1* is only expressed in meristems and developing vascular bundles and belongs to a gene family that plays diverse roles in plant development (Sinha and Hake 1990). At the stage that meristematic cells become designated to form organs, they stop expressing *KN1*. However, outgrowths are formed on leaf blades in *kn1* mutant plants, probably by dedifferentiation. Expression of *kn1* in only the middle mesophyll–bundle sheath layer of the leaf induced the formation of tissue knots in all other cell layers (Jackson et al. 1994). Its site-limited expression thus leads to developmental reactions in neighboring cells and is indicative of KN1 trafficking across cellular boundaries. Immunolocalization studies established that KN1 was present in cells where *KN1* mRNA was lacking (Lucas et al. 1995). Apparently, KN1 has the capability of binding to the molecular apparatus of PDs so that it may move from cell to cell in a similar fashion as viral MPs. Rapid PD-mediated movement of FITC-labeled KN1 to adjacent cells, even in other plant species, gave credence to this conclusion (Lucas et al. 1995). Not much later, PD trafficking of two other transcription factors (DEFICIENS and GLOBOSA), both involved in flower development, was discovered (Perbal et al. 1996). These factors act in a non-cell-autonomous fashion after moving from the inner layer to the epidermal cells of the inflorescence meristem.

The next emerging question was whether KN1, in analogy to viral MPs, is able to traffic its own mRNA. It was determined that KN1 is capable of recognizing, binding, and trafficking its mRNA (Lucas et al. 1995), even in a more specific manner than, for example, CMV MP (Vaquero et al. 1994). The previous findings led to the postulate that supracellular control genes control cellular processes beyond the cell boundary (Mezitt and Lucas 1996). The functional resemblance between KN1 and viral MP (both are able to mediate PD trafficking of their own mRNA) raised questions regarding the origin of virus trafficking.

### *11.7.6 Opening States of Plasmodesmata*

Data on the gating of PDs presented a maze of contrasting observations by the end of the 1990s (see Sect. 11.7.2). Initially, the molecular exclusion limit of PDs was assumed to be a standard 1 kDa (Tucker 1982; Goodwin 1983; Terry and Robards 1987; Tucker et al. 1989). However, testing the functional diameters of PDs between cells types other than mesophyll revealed molecular exclusion limits of up to 7 kDa for PDs between *Nicotiana* trichome cells (Waigmann and Zambryski 1995), 10 kDa for PDs between root cells (Wang and Fisher 1994), at least 3 kDa

for PPUs in Cucurbita (Kempers et al. 1993), at least 10 kDa for PPUs in Vicia (Kempers and van Bel 1997), and 30 kDa for PPUs in *Arabidopsis* and *Nicotiana* (Imlau et al. 1999). The molecular exclusion limits, in particular those measured using dextrans, might be appreciably larger if their hydration shell is taken into account (Wang and Fisher 1994; Böckenhoff et al. 1996).

An additional complication to the contradictory findings on molecular exclusion limits was the claim by Radford and coworkers (1998) that fluorochrome injection via microcapillaries affected the molecular exclusion limit (see Sect. 11.5.3). Wounding inflicted by the penetrating tip gave rise to $Ca^{2+}$ release into the cytoplasm, inducing immediate callose synthesis and deposition around the PD neck areas (Radford et al. 1998). Wound-induced shut-off of PDs could explain why the fluorescent halos around the site of injection remained limited to the distance of a few cells from the site of injection (e.g., Goodwin 1983). Injection of $Ca^{2+}$ ions (see Sect. 11.4.4) strongly affected cell-to-cell transport of fluorochromes (Erwee and Goodwin 1983; Baron-Epel et al. 1988; Tucker 1990). It was therefore to be expected that PDs having callose collars would display lower molecular exclusion limits than PDs without. To add to the confusion, PDs of mesophyll cells without neck constrictions had smaller functional diameters than those between trichome cells with apparent neck collars in *Nicotiana* (Waigmann et al. 1997).

A further confounding issue (see Sect. 11.7.1) was the seeming involvement of metabolic energy in PD closure (Tucker 1993; White et al. 1994; Cleland et al. 1994; Wright and Oparka 1997), although the evidence was not entirely equivocal (Zhang and Tyerman 1997). These contrasting observations on the size and status of the PD diameter led Schulz (1999) to hypothesize three PD configurations: (1) an actively closed state in which callose deposition plays a role, (2) a passively opened "resting" state, and (3) an actively opened state in which motor proteins may be involved. This proposal was generally accepted as a guideline during subsequent years (e.g., Lucas et al. 2009).

### *11.7.7 The 1990s as the Era of Shifting Paradigms in Plasmodesmal Research*

By the turn of the century, the structural and functional understanding of PDs had undergone fundamental changes, as documented in a number of reviews (Ding 1997; Ghoshroy et al. 1997; McLean et al. 1997; Kragler et al. 1998; Ding 1998). PDs were no longer regarded as narrow passive channels through which only "micromolecules" (<1 kDa) could diffuse, but rather as highly dynamic and adjustable pores that allowed selective transfer of macromolecules. The notion that PD corridors could allow macromolecular trafficking paved the way for new avenues for research. One of the most exciting novelties during the following years was the recognition that all sorts of macromolecular signals could be released via

the PPUs from the companion cells into the sieve elements (Lee and Cui 2009; Ham and Lucas 2017). These systemic signals not only included an arsenal of sieve element proteins, but also RNA molecules in diverse forms (mRNA, siRNA, sRNA, nucleoprotein complexes). This exciting discovery triggered new waves of research. Most of the ingredients of today's PD research emerged within less than a decade. These ideas and concepts were further extended and refined during the following years (see Sect. 11.8).

Because the history of PD research is the focus of this chapter, modest attention is devoted to recent developments in PD research. All recent discoveries are readily accessible via a broad palette of reviews (Lee and Cui 2009; Lucas et al. 2009; Benitez-Alfonso et al. 2010; Faulkner and Maule 2011; Niehl and Heinlein 2011; White and Barton 2011; Maule et al. 2011; Wu and Gallagher 2012; Brunkard et al. 2013; de Storme and Geelen 2014; Sager and Lee 2014; Kumar et al. 2015; Tilsner et al. 2016; Brunkard and Zambryski 2017; Ham and Lucas 2017). Only a few lines of research are concisely discussed here, because it is virtually impossible to encompass all recent developments in one review.

## 11.8 Some Concepts, Highlights, and Issues in Current PD Research

Transfer through PDs has an indispensable impact on a broad range of developmental and physiological processes and on the dissemination of viral infections. All effects essentially depend on the molecular structure and associated functioning of PDs. Therefore, recent developments in PD research are discussed with a focus on the molecular mechanisms of PD passage. The consequences for developmental biology and viral spread are not discussed, but the physiological impact of collective PD efforts is exemplified by a short discussion of the significance of PDs for phloem physiology.

### *11.8.1 Plasmodesmal Architecture and Composition: Shaping and Maintenance of the Plasmodesmal Substructure*

#### 11.8.1.1 Lipid Backbones of Plasmodesmata Membranes

Over the last 15 years, much knowledge has been gained on the exceptional architecture and membrane composition of plasma membranes and desmotubular membranes in PDs (Tilsner et al. 2016). As mentioned before (see Sect. 11.7.2; Kikuyama et al. 1992), desmotubules are absent in PDs formed during phragmoplast-dependent cell division in Chlorophycean taxa. Hence, it was

proposed that PD formation in division walls is not the inevitable consequence of phragmoplast-mediated cell division, but rather a mechanism that evolved in land plants (Brunkard and Zambryski 2017). Primary PDs on division walls are thought to be deployed randomly (Faulkner et al. 2008). Such PDs are often modified during developmental progression or cellular specialization, which yields a vast morphological variety of PDs (Fig. 1 in Sager and Lee 2014). At sites where PDs are being created, both plasma membrane and ER membrane are transformed into narrow tubular structures with an extreme degree of bending. Moreover, the plasma membrane is curved in the neck region at an angle of more than 90° (positive curvature), which changes into a slightly negative curvature along the cytoplasmic sleeve (Tilsner et al. 2016).

Membrane curvature is largely caused by asymmetric distribution of lipids in the membrane leaflets (McMahon and Boucrot 2015). The geometry of a lipid layer depends on the relative size of the polar heads and the acyl chain. Lipids with large heads and a slender, straight acyl chain have an inverse conical shape that causes a negative membrane curvature (McMahon and Boucrot 2015). Phospholipids, such as phosphatidylinositol phosphates or sphingolipids, possess large polar heads. Lipids with small polar heads and acyl chains with kinks (due to unsaturated bonds) have a conical shape and cause a positive membrane curvature (McMahon and Boucrot 2015). Analogous lipids may be assembled in microdomains to sustain the membrane curvature (Tilsner et al. 2016). The curved membrane is fortified by insertion of all sorts of sterols and stabilized by the apposition of internal or external protein scaffolds, such as BAR domains (Peter et al. 2004). Furthermore, positive curvatures are maintained by bonds between the lipid backbone and proteins with amphipathic helices (McMahon and Boucrot 2015), reticulons (Tolley et al. 2010; Fernandez-Calvino et al. 2011; Knox et al. 2015) and synaptotagmins (Levy et al. 2015; Perez-Sancho et al. 2015). Obviously, membranes participating in PD architecture must contain a variety of microdomains to meet the spatial requirements of the permanent and extreme degrees of bending and to execute a number of functions within a minimum amount of space. Such local membrane specialization implies that the lipid composition of the plasma membrane lining the PDs must differ from that outside the PD area. Membrane fractions enriched in PD components do indicate a lipid composition that is distinct from the other parts of the plasma membrane (Grison et al. 2015).

#### 11.8.1.2 Proteomes Associated with Plasmodesmata

After countless attempts extending over many years, isolation procedures can now successfully yield stable and reliable PD proteomes (Maule et al. 2011). First, several proteins and protein complexes were detected that stabilize and maintain the PD architecture (see Sect. 11.8.1.1). Synaptotagmins form protein tethers between ER and plasma membrane, keep the desmotubule in place, and enable lipid exchange between the membrane partners (Levy et al. 2015; Perez-Sancho et al. 2015). Synaptotagmin A, for instance, is physically associated with the

reticulons RTLNB3 and RTLNB6 and appears to be engaged in targeting and displacement of viral MPs (Lewis and Lazarowitz 2010; Levy et al. 2015). Reticulons are attached via two loop insertions in the ER membrane (Tolley et al. 2010; Fernandez-Calvino et al. 2011) in order to shape the extreme curvature of the desmotubule (Kriechbaumer et al. 2015; Knox et al. 2015), in cooperation with THD3-like 2 (Lee et al. 2013).

Likewise, the plasma membrane curvature is maintained by tetraspanins (Fernandez-Calvino et al. 2011) that possess four membrane-spanning domains with two extracellular loops, and terminal ends at the cytosolic side. Receptor-like kinases (RLKs; Jo et al. 2010; Fernandez-Calvino et al. 2011) are situated between the tetraspanins. Toward the neck regions, the inner leaflet of the plasma membrane is covered by remorin microdomains (Raffaele et al. 2009) that colocalize with transmembrane RLKs, a group of receptors such as the chitin-receptor LYM2 and the flagellin-receptor FLS2 (Faulkner et al. 2013). Furthermore, the plasma membrane contains transmembrane receptor-like proteins collectively named PD-localized proteins (PDLPs; Thomas et al. 2008; Amari et al. 2010).

The PDLP family includes crucial regulators of PD transport that are engaged in antipathogenic responses (Lim et al. 2016; Otero et al. 2016) and in targeting specialized MPs that irreversibly transform PDs into tunnels for the passage of virus particles (den Hollander et al. 2016; Xie et al. 2016). It has been suggested that the presence of the receptors LYM2 and FLS2 at PDs (Faulkner et al. 2013) is merely coincidental (Brunkard and Zambryski 2017). Pathogenic signals perceived by these receptors at non-PD plasma membrane sections lead to NPR1 production and NPR1 binding to PLDP5, resulting in callose deposition (Lee et al. 2011; Wang et al. 2013). In the neck region, remorin-enriched domains at the inner plasma membrane leaflet are accompanied by glycosylphosphoinositide (GPI)-anchored proteins, such as PD-localized callose-binding proteins (PDCBs; Simpson et al. 2009), PD-localized β-glucanases (PDBGs; Levy et al. 2007; Rinne et al. 2011), and possibly the CalS holoenzyme complex (responsible for callose synthesis) attached to the outer leaflet. The accumulation of GPI-anchored proteins at PDs could coincide with a specific lipid composition in the PD area (Levy et al. 2007; Simpson et al. 2009).

Although the PD association of the MPs actin (White et al. 1994; Ding et al. 1996; Blackman and Overall 1998) and myosin (Radford and White 1998; Blackmann and Overall 1998; Reichelt et al. 1999; Baluska et al. 2001) was established some 20 years ago (see Sect. 11.5.1), we are still searching for their precise location, structural interrelations, and function. In the light of the limited space in the cytoplasmic sleeve, it is questionable whether myosin and actin both fit inside this narrow intermembrane space (Bell and Oparka 2011; Tilsner et al. 2011). If not, myosin and actin may be involved in the entering and exiting of macromolecules into and from the PD corridor. If both are present inside the sleeve, it is uncertain whether they form a conveyer belt for transfer of macromolecules or provide a frame of buttresses that prevent collapse of the cytoplasmic sleeve (see Sect. 11.8.2.3).

In most PD models, myosin chains are thought to be arranged as a row of horizontally oriented spokes spiraling around the desmotubule (like a spiral staircase), bridging the space between ER membrane and plasma membrane. The myosin heads are linked to filamentous actin that is tethered to the plasma membrane (Sager and Lee 2014) or to actin filaments spiraling around the desmotubule, while the cargo ends are connected to cargo complexes at the inner plasma membrane face (Oparka 2004). Both arrangements could accommodate the spatial constraints of PDs, provided that the actin filaments are flat (Dominguez and Holmes 2011) and partly incorporated into the inner membrane leaflets (Gicquaud and Wong 1994). The uncommon class VIII myosin AT M1 localized to PDs (Haraguchi et al. 2014) could possibly act as a structural consolidation to keep the PD sleeve open. The most convincing argument in favor of a support function of myosin VIII is its high affinity for actin, in combination with a low actin-sliding capacity and a slight ATPase activity (Haraguchi et al. 2014).

## *11.8.2 Narrowing of the Plasmodesmal Passageway*

### 11.8.2.1 Plasmodesmal Constriction by Callose

Regulation of callose (β-1,3-glucose chains) deposition around the PD neck regions (Radford et al. 1998) is regarded as the key mode for constriction of the functional PD corridor (de Storme and Geelen 2014). This claim is supported by an inverse correlation between PD permeability and the size of callose depositions around PD necks (Tucker and Boss 1996; Holdaway-Clarke et al. 2000; Zavaliev et al. 2011; Tilsner et al. 2016), although the permanence of callose collars is subject to debate (see Sects. 11.4.4, 11.5.3 and 11.7.6; Levy and Epel 2009). Callose synthesis is regulated by two protein families with opposite enzymatic functions (reviewed by Zavaliev et al. 2011; de Storme and Geelen 2014; Kumar et al. 2015; Tilsner et al. 2016): plasma transmembrane β-1,3-glucan synthases or callose synthases (GSLs, a multigene family) and β-1,3-glucanases (BGs, a family of about 50 members) attached to the outer face of the plasma membrane. GSLs are probably incorporated into complex transmembrane protein agglomerates, named CalS holoenzyme complexes (Amor et al. 1995; Hong et al. 2001). GSL8 is involved in callose deposition in PDs in a wide variety of tissues (Guseman et al. 2010), and *GSL12* is held responsible for adjusting the functional diameter (Vaten et al. 2011). Three BGs (PDBGs) are associated with PD regions (Zavaliev et al. 2013; Benitez-Alfonso et al. 2013), where they are probably anchored to lipidic glycophosphatidylinositol heads (Gaudioso-Pedraza and Benitez-Alfonso 2014).

As mentioned in Sect. 11.4.4 (Levy and Epel 2009), it is unclear whether the callose deposition around the PD necks observed in damaged sections or tissues is an artifact of injection, fixation, or cutting procedures (Radford et al. 1998). There may always be a residual callose collar present around sieve pores as a relic of their correct formation (Evert 1990; Barratt et al. 2011), but the amount of callose in

damaged specimens probably exceeds the *in vivo* situation. It is beyond doubt that callose is involved in constriction of the PD pathway, as the callose collars fluctuate in size and correlate negatively with PD permeability (Lee et al. 2011; Rinne et al. 2011; Vaten et al. 2011).

Because increased intracellular $Ca^{2+}$ narrows the symplasmic corridor (Erwee and Goodwin 1983; Baron-Epel et al. 1988; Tucker and Boss 1996) and callose synthesis is presumed to be $Ca^{2+}$ dependent, the cytosolic $Ca^{2+}$ level in PD regions may control the functional diameter and transport capacity of the PD passageway. Although compelling molecular evidence appears to be lacking that cytosolic $Ca^{2+}$ acts as the motor of callose synthesis through $Ca^{2+}$ binding sites at the CalS complexes, a striking number of $Ca^{2+}$-regulatory instruments are associated with the PD area.

The following are some of the candidates for $Ca^{2+}$ regulation:

(a) Unilaterally branched PPUs between sieve elements and companion cells colocalize with $Ca^{2+}$ hotspots (Furch et al. 2009; Hafke et al. 2009) created by aggregates of $Ca^{2+}$-permeable ion channels in plasma and ER membranes. There is ample circumstantial evidence that such a configuration also occurs near "normal" PDs, given their evolutionary relationship. In response to appropriate stimuli, an abruptly rising $Ca^{2+}$ level can be amplified by $Ca^{2+}$ release from the nearby ER cisterna via $Ca^{2+}$-stimulated $Ca^{2+}$-permeable channels (CICR channels) so that the local cytosolic $Ca^{2+}$ concentrations can rapidly rise to excessive values (Hafke et al. 2009).
(b) Reduced PD trafficking in wild-type plants treated with oxidants (Benitez-Alfonso et al. 2009) and in *Arabidopsis* mutants defective in glutathione production (Cairns et al. 2006) indicates that oxidants stimulate callose production. It is unclear whether the redox state directly interacts with the GSLs or whether the reactive oxygen species stimulate gating of local $Ca^{2+}$-permeable channels, as shown for hydrogen peroxide (Lecourieux et al. 2006).
(c) Salicylic acid produced as a response to infections exerts control on PD closure via NPR1 that binds to PDLP5, a transmembrane receptor-like protein in the plasma membrane toward the neck region, giving rise to callose synthesis (Lee et al. 2011; Wang et al. 2013).
(d) Several $Ca^{2+}$-binding catalytic proteins such as calmodulin (Zavaliev et al. 2011), centrin-like proteins (Blackman and Overall 1998), and calreticulin (Baluska et al. 1999, 2001; Reichelt et al. 1999) are localized to PD orifices.
(e) Protein anchors, synaptotagmins that act as membrane contact sites (Henne et al. 2015) between ER membranes and plasma membrane in the PD region, also function as $Ca^{2+}$ sensors and are engaged in $Ca^{2+}$ homeostasis (Burgoyne et al. 2015; Lahiri et al. 2015).

#### 11.8.2.2 Electrical Conductivity and Callose Deposition and Their Consequences for Symplasmic Organization

The administration of electrical pulses has often been employed as a means to measure PD conductance (see Sect. 11.3.2 and Yang et al. 1992; Holdaway-Clarke et al. 1996; Lew 1996). Electrical currents are also natural phenomena triggered by abrupt changes in intact plants under natural conditions. It is known that PD passage (including sieve pores) of innate electrical currents has a remote impact on distant physiological processes such as photosynthesis (Vodeneev et al. 2016). Several stimuli and consequent local or systemic electrical potential waves (van Bel and Ehlers 2005) may exert effects on symplasmic organization that have been rarely considered. Strong local stimuli trigger long-distance electrical potential waves, most probably propagating along the sieve tubes (Furch et al. 2007; Hedrich et al. 2016), that are associated with tidal waves of cytosolic rises in $Ca^{2+}$ and reactive oxygen species in successive sieve elements (Gilroy et al. 2014; van Bel et al. 2014; Choi et al. 2016; Hedrich et al. 2016).

Passing waves of $Ca^{2+}$ influx induce temporary (up to 3 h) protein- and callose-mediated closure of sieve pores (Furch et al. 2007, 2009, 2010; Hafke et al. 2009) and PPUs (Furch et al. 2009). Because electrical waves, accompanied by $Ca^{2+}$ influx, spread in a lateral direction along the path (Rhodes et al. 1996), it was speculated that PDs between cells adjoining the vascular conduits in question also close as a result of a rise in cytosolic $Ca^{2+}$ (van Bel et al. 2014). Transient PD and sieve pore closure would confer a temporary autonomy to these cells. PD closure may prevent exchange of correcting and interfering agents so that these cells can execute genetic and metabolic programs that deviate from those in neighboring cells (van Bel et al. 2014).

The question arises whether electrical currents are able to pass the space between the appressed ER and plasma membranes in the neck region. If the sealing is not perfect, electrical signals may creep through the chinks during PD closure. Even if the cytosolic sleeve is hermetically closed, the desmotubular corridor could provide a pathway for electrical conductance given the minute size of ions and electrons.

#### 11.8.2.3 Involvement of Plasmodesmal Constriction in Plant Immunity

In the hypersensitive response, infected cells are killed by apoptosis to raise a barricade against pathogenic attack and to prevent further progress of the disease. In response to numerous fungal infections, apoptosis is often preceded by fortification of the cell wall by the formation of a papilla and followed by the closure of PDs giving access to the adjacent cells, as exemplified by the reaction of *Hordeum* epidermal cells to *Blumeria graminis* (An et al. 2006a, b). In both responses, callose deposition is involved. A considerable part of the papilla is made up of callose delivered by exocytosis of multivesicular bodies (An et al. 2006a), whereas permanent closure of PDs is executed by deposition of a callose collar around PDs

(An et al. 2006b). Constriction events were discovered for several types of pathogenicity (Lee and Lu 2011; Li et al. 2011a; Lim et al. 2016; Cui and Lee 2016) and are probably related to PDLPs (Lim et al. 2016; Otero et al. 2016). As an emerging picture for the molecular basis of PD-mediated defense, elicitors of extracellular phytopathogens are perceived by membrane-bound receptors. Downstream products of defense cascades interact with PDLPs, which, in turn, trigger the activity of CalS holoenzyme complexes (see Sect. 11.8.2.1). As an example of this type of defense, NPR1, the downstream product of an SA-induced cascade, binds to PDLPs at the neck region, which induces callose deposition (e.g., Lee et al. 2011; Wang et al. 2013). A similarly detailed description of the order of events is not yet available for the confinement of viral infections by PD constriction (Li et al. 2011b).

#### 11.8.2.4 Plasmodesmal Occlusion by Proteins?

In contrast to reviews that assign a key role to callose deposition in PD closure (e.g., Tilsner et al. 2016), doubts have been raised concerning the idea that callose is the only or central regulator of PD closure (e.g., Sager and Lee 2014). Sager and Lee (2014) argued that callose synthesis would require several minutes for completion of a PD shut-down, with reference to pertinent reports (Radford and White 1998; Bilska and Sowinski 2010). Circumstantial evidence is supportive of a time lapse between stimulus and callose-executed PD closure. First, induction of callose synthesis may depend not only on $Ca^{2+}$, but also on an endogenous activator fosforylglucoside that must be released first from the vacuole, which may be time-consuming (Ohana et al. 1993). Second, callose build-up requires a considerable period until completion. Callose deposition around the sieve pores triggered by remote wounding reaches its maximum after 20 min in sieve elements of intact *Vicia faba* plants (Furch et al. 2007).

The slow callose build-up (a matter of minutes) is preceded by an immediate (within seconds) $Ca^{2+}$-dependent sieve pore occlusion by forisomes (Furch et al. 2007), which are highly ordered protein bodies composed of sieve element occlusion proteins. These proteins also occur as loosely organized filaments in other eudicotyledonous families (Rüping et al. 2010). Because sieve pores are ontogenic descendants of PDs (Evert 1990), proteins in the vicinity of PDs may react to an abrupt increase in the $Ca^{2+}$ level with similar coagulation processes, resulting in immediate and temporary PD plugging prior to more permanent constriction of the cytoplasmic corridor by callose. In the same line of reasoning, Schulz (1999) pointed at an unknown electron-dense material in the PD region, detected during tissue domain development (Kwiatkowska and Maszewski 1986; Ehlers and Kollmann 1996) and in plasmolyzed tissue (Tilney et al. 1991; Oparka et al. 1994; Schulz 1995). Therefore, it may be worthwhile to investigate whether PD closure is a biphasic event, as is the case for sieve pore occlusion (Furch et al. 2007, 2010).

### 11.8.3 Dilation of the Plasmodesmal Passageway for Macromolecular Trafficking

The mechanisms responsible for dilation of the PD passageway must be entirely different from those of callose-mediated closure. It is a common opinion that the cytoskeleton is involved in enlargement of the functional diameter and passage of macromolecules through PDs (White and Barton 2011). Disruptors of actin and mysosin polymerization have a clear impact on PD widening and macromolecular passage, but the effects vary widely depending on both the type of PDs and the plant species (summarized in White and Barton 2011). Despite a range of sophisticated techniques, such as superresolution microscopy (Fitzgibbon et al. 2010) and electron microscopy tomography (Bell and Oparka 2011), we do not have the faintest idea how cytoskeletal components are linked to PD entry and whether they traverse the entire PD corridor (White and Barton 2011).

Inside PDs, tropomyosin-like molecules (Faulkner et al. 2009) could prevent the disassembly of tropomysosin-associated actin (Staiger et al. 2009). It should be noted that tropomyosin is not absolutely necessary for myosin movement along actin filaments, but the speed of progression is appreciably lower without tropomyosin (Higashi-Fujime and Nakamura 2009). Tropomyosin may be regarded as a stabilizer, not as an accelerator (White and Barton 2011), because it usually occurs in the vicinity of highly stable actin bundles (Gupton et al. 2005; Faulkner et al. 2009) and even more so because it is hard to conceive how myosin could move along an actin filament in the limited space of the cytoplasmic sleeve.

Potentially, some of the numerous actin-binding proteins (Deeks et al. 2012) are also engaged in PD trafficking. The actin-binding protein Arp2/3, which is mainly found in the neck region, is virtually absent inside the PD corridor (van Gestel et al. 2003). The fact that Arp2/3 binds weakly to tropomyosin-complexed actin (Gupton et al. 2005) hints at a tropomyosin-stabilized actin inside the sleeve. In conclusion, a stabilizing function of the cytoskeleton inside PDs is in agreement with the presumptive properties of mysosin VIII (see Sect. 11.8.1.2 and Haraguchi et al. 2014; Tilsner et al. 2016). Given their mechanical properties, it is possible that myosin units represent the spokes inside the PD and that their heads are connected to linear actin embedded in the plasma membrane (White and Barton 2011). How this scaffold, stabilized by myosin buttresses, becomes erected to dilate the PD corridor remains a matter of debate.

### 11.8.4 Mechanism(s) of Macromolecular Transfer Through Plasmodesmata

The mechanisms of dilation and associated macromolecular transfer are still largely unresolved. Macromolecular transfer is fundamentally different through immature (between meristematic cells) and mature (between fully differentiated cells) PDs,

which may be related to their ultrastructure. The ultrastructure of PDs between immature cells, such as early embryonic or meristematic cells, and mature cells, such as mesophyll cells, is entirely disparate (Ding et al. 1992b; Moore et al. 1992). With increasing PD maturity, a substructure emerges within the symplasmic sleeve (Ding et al. 1992b), which is postulated to be a scaffold of motor proteins (see Sects. 11.5.1 and 11.8.2.4). In addition, the deposition of callose collars often gives rise to neck constrictions in the course of PD maturation (Ding et al. 1992b).

Despite the tightly appressed appearance of the membranes of immature PDs (Ding et al. 1992b), their functional diameter is generally larger than that of mature PDs. Several noninnate molecular constructs up to 70 kDa in size freely move between sink cells in leaves (Oparka et al. 1999; Itaya et al. 2000; Crawford and Zambryski 2001) and roots (Stadler et al. 2005). Similar large exclusion limits were found for PDs in young embryos (Kim et al. 2005). The free mobility of macromolecular constructs in the 70–80 kDa range between sink cells declines with the progression of cell and PD development to values between 1 and 5 kDa (Duckett et al. 1994; Oparka et al. 1999; Crawford and Zambryski 2001). Proteins smaller than 70 kDa are able to move freely between meristematic cells at a stage when positional information is crucial for cell differentiation. However, free trafficking of all sorts of larger molecules seems to conflict with the fine tuning required during the predifferentiation period.

The molecular limit of PDs also drops dramatically during embryogenesis of *Arabidopsis*, for which the *IES1*- and *IES2* genes are responsible (Kim et al. 2002). Because the reduction in permeability coincides with establishment of the PD scaffold (Ding et al. 1992b), *IES* genes might be engaged in installation of the scaffold.

Remarkably, viral MPs do not target immature PDs, which prevent trafficking of viral genomes (Ding et al. 1992a; Itaya et al. 1998). Docking of viral MPs to mature PDs is ascribed to the presence of binding sites that are absent in immature PDs. MP docking at PDs with low molecular exclusion limits enables nonselective movement of macromolecules (Wolf et al. 1989; Lapidot et al. 1993; Vaquero et al. 1994; Oparka et al. 1997). Dilation induced by innate chaperones only allows highly specific macromolecular trafficking. Noninnate macromolecules (e.g., GUS) can only pass mature PDs linked to the chaperone (Waigmann and Zambryski 1995).

According to long-standing models, viral MPs and several transcription factors bind to a receptor or docking protein at the PD entrance, which allows two modes of macromolecular transfer (Lucas et al. 2009): (1) Several viral MPs, but not all (e.g., Waigmann and Zambryski 1995; Niehl and Heinlein 2011), bind to a docking protein located at the PD orifice, resulting in dilation of narrow PD corridors to nonselective tunnels through which macromolecules up to 70 kDa in size can pass passively (Lucas et al. 2009). Dilation may be the result of several simultaneous processes. The MP of tobacco mosaic virus triggers breakdown of callose, depolymerization of the actin filaments, and reduction of cell wall rigidity so that the PD opens up and allows nonselective passage of molecules (Niehl and Heinlein 2011). Speculatively, this is not the only mechanism of dilation for passive passage of macromolecules. (2) Other MPs (Niehl and Heinlein 2011) and the innate non-cell-autonomous proteins and

their mRNAs attach to a docking protein, unfold, and slide through the PDs (Lucas et al. 2009) with or without the aid of motor proteins.

A remaining burning question is how cytoskeletal components (see Sects. 11.8.1.2 and 11.8.3) fit into current models of PD trafficking (Lucas et al. 2009). This question also pertains to the targeting of PDs by macromolecules and to the connection of intracellular sorting systems with cytoskeletal structures at the orifices and inside the cytoplasmic sleeves. Based on the premise that MPs highjacked the mechanisms of innate macromolecular trafficking during evolution, non-cell-autonomous proteins and MPs share intracellular transfer features. The diverse strategies of viral MPs to target and pass PDs (Niehl and Heinlein 2011) make it plausible that there is a wealth of possibilities for targeting PDs and passage of non-cell-autonomous proteins through PDs.

The discussion on macromolecular trafficking is further complicated by the fact that many PDs deviate from the proclaimed 1 kDa standard size for nonselective trafficking in mature PDs (see Sects. 11.7.2 and 11.7.3). Instead of a molecular exclusion limit of 1 kDa in staminal trichomes of Setcreasea (Tucker 1982; Tucker et al. 1989) and nectary trichomes of *Abutilon* (Terry and Robards1987), PDs in leaf trichomes of tobacco have an exclusion limit of 7 kDa (Waigmann and Zambryski 1995). PPUs between companion cells and sieve elements exhibit permanent nonselective molecular exclusion limits of at least 10 kDa, and up to 70 kDa (Kempers and van Bel 1997; Imlau et al. 1999; Stadler et al. 2005). Moreover, PDs at the vascular border in sink tissues seem to have the capacity to enlarge their functional diameters in response to variable intercellular turgor gradients (Wang and Fisher 1994; Patrick et al. 2015).

Given the variety in molecular exclusion limits, morphological traits, and functions of PDs, the extent to which macromolecular cell-to-cell signaling and signaling by phloem-mobile macromolecules depend on similar or entirely different modes of PD targeting and trafficking through PDs is still a mystery. Key events in macromolecular trafficking such as the modes of intracellular transfer to PDs and the entrance, passage, and exit mechanisms involved may vary between PDs. Because phloem physiology is intertwined with the functioning of the diversity of PDs (Patrick et al. 2015), the role of PDs in phloem biology is briefly discussed below.

### *11.8.5 Roles of Plasmodesmata in Phloem Physiology: Loading, Translocation, and Unloading of Photosynthates*

In minor veins of apoplasmically phloem-loading species, the virtual absence of PDs at the interface between mesophyll and companion cells (Gamalei 1989) creates a borderline between two symplasmic domains (see Sect. 11.4.2). Consequently, sucrose that leaks from the mesophyll into the apoplasm can be

accumulated to high concentrations by carriers located in the sieve element plasma membrane. Because perfect isolation by the absence of PDs would provide even better conditions for sucrose accumulation, the role of the scarce PDs inevitably comes to mind. Perhaps, local cell integration requires exchange of electrical information via a circuit provided by a few narrow PDs. In symplasmatically phoem-loading species, PDs between mesophyll (sheath) and companion cells act as valves through which building blocks of the raffinose family of sugars diffuse to the companion cells (see Sect. 11.4.2). Raffinose family sugars are fabricated in the companion cells, but fail to diffuse back to the mesophyll because their molecular size exceeds the exclusion limit of the PDs (Beebe and Turgeon 1992).

The functional counterpart of a symplasmic intermission is a structural interruption in apoplasmic continuity, forcing molecules to follow a symplasmic pathway. This situation is created by the Casparian strips in the root endodermis (Schreiber 2010) and by suberin layers in the walls between the mestome sheath and vascular parenchyma in monocotyledonous veins (Botha et al. 1982, 2005). It is anticipated that further blockades put up by less conspicuous wall barriers will be discovered at any site where symplasmic transfer is necessary, such as at the exchange interfaces in $C_4$ plants (Niyogi et al. 2015). The intensive exchange of $C_4$ metabolites raises questions regarding PD trafficking at this interface. Whether the solutes diffuse along their gradients in opposite directions through the same PDs or whether two types of PD exist, each specialized for unidirectional polar transport (see Sect. 11.7.1), remains to be investigated.

Under natural conditions, sink demand is generally larger than source production (low source/sink relationship). Under these conditions, the application of carboxyfluorescein diacetate reveals a symplasmic intermission at the interface between sieve element–companion cell complexes and phloem parenchyma cells in the transport phloem of a number of species, including *Vicia faba*, *Solanum lycopersicum*, *Cucurbita pepo*, and *Ocimum basilicum* (Hafke et al. 2005). Pruning bean plants to an extremely low source/sink ratio led to a similar containment of carboxyfluorescein and $^{14}$C-sucrose in sieve tubes (Patrick and Offler 1996). In contrast, carboxyfluorescein and $^{14}$C leaked *en masse* from the sieve tube–companion cell complexes in bean plants with high source/sink ratios (Patrick and Offler 1996). This switch suggests strict regulation of the functional PD passageway at the borderline between sieve element–companion cell complexes and phloem parenchyma cells.

A tentative explanation for these phenomena (Patrick 1997) is that the turgor gradient over this border is usually high, leading to PD shutdown (see Oparka and Prior 1992). Plasmodesmograms revealed low PD frequencies at this border in transport phloem of *Vicia faba, Zinnia elegans, Lythrum salicaria*, and *Cucurbita maxima* (Kempers et al. 1998). The paucity of PDs (Kempers et al. 1998) and a narrow functional diameter may maintain a steep osmotic gradient at this interface, establishing a high turgor gradient. Under high source/sink ratios, excess photosynthate leaks from the sieve tubes through the plasma membranes and is retrieved by the phloem parenchyma cells (Hafke et al. 2005). As a result, the turgor gradient levels off and the PDs open up (Patrick 1997; Patrick et al. 2015).

Similarly, PDs at the same borderline are considered to be regulatory bottlenecks in the unloading pathway, as phloem unloading generally occurs by diffusion through PDs (Wang and Fisher 1994; Fisher and Oparka 1996; Patrick 1997; Schulz 1999). These PDs are regarded as the control gates in photoassimilate unloading from the release phloem in sinks. They are located at an interface where pressure differentials of more than 200 kPa are maintained to provide sufficient diffusion rates (Fisher and Wang 1995). Functional PD diameters increase when the pressure difference between the sieve element–companion cell complexes is reduced by bathing root tips in an osmoticum mimicking shortage of photosynthate by a drop in turgor of the sink cells (Schulz 1995). The sugar concentration in sink cells almost equals the concentration in the sieve tubes (Fisher and Wang 1995). Because dilation of the PD pathway is directly related to the efflux of $^{14}$C-photosynthate from the sieve tubes (Schulz 1994, 1995), it seems obvious that the rate of phloem unloading depends on the functional diameter of the PDs between companion cells and phloem parenchyma, which is adjustable to the sink demands. These PDs have additional special properties: They lack the machinery to traffic the genome of phloem-limited viruses. Hence, poleroviruses (Peter et al. 2009), luteoviruses (Brault et al. 2011), and most geminiviruses are unable to escape from the sieve element–companion cell complexes.

Phloem unloading follows a diffusive symplasmic pathway along the concentration gradient in small sinks, whereas large sinks exhibit more complex patterns of phloem unloading (Patrick et al. 2015). PDs play a vital role in switching the mode of unloading in storage sinks (e.g., potatoes) or in large reproductive sinks (e.g., grapes). Phloem unloading follows an apoplasmic path in potato stolons until the onset of starch accumulation. A drastic reorganization of PD connectivity then transforms the mode of unloading into the symplasmic mode (Viola et al. 2001). On the other hand, phloem unloading in grape berries was initially symplasmic, but adopted an apoplasmic mode during the major phase of sugar accumulation (Zhang et al. 2006). The rationale for this switch probably lies in the changing concentration gradients between sieve elements and sink cells: As long as the sugar concentration in sieve elements is higher, the symplasmic mode of unloading is the most effective. Conversely, accumulation of sugars in sink cells, leading to a higher concentration, requires apoplasmic phloem loading. PD gating thus confers flexibility in regulating nutrient fluxes as sink function alters across development (Patrick et al. 2015).

Phloem physiology is of paramount importance for long-distance signaling. Multiple phloem-mobile transcripts, fabricated in companion cells, are released into the sieve tubes for systemic distribution and have a remote impact on development and physiology (Ham and Lucas 2017). It has been demonstrated that phloem-mediated transport of macromolecules is involved in shaping leaf morphology (Kim et al. 2001), flowering (Jaeger and Wigge 2007; Li et al. 2011a) and many other developmental and physiological processes. The HY5 transcription factor produced during the day, for example, promotes distant nitrogen uptake from the soil and balances carbon fixation (Chen et al. 2016). It is a matter of fierce debate whether macromolecular trafficking through PPUs toward the sieve

elements is selective or not. Evidence was presented that size, abundance, and subcellular localization in companion cells, and not the chemical physicochemical properties of the components, are decisive factors for the delivery of proteins to the phloem stream (Paultre et al. 2016) and, hence, that macromolecular trafficking through PPUs is nonselective. Moreover, only some of the proteins in the sieve-tube sap have a non-cell-autonomous function. For the distinction between non-cell-autonomous proteins and others, novel protocols have been developed (Besnard et al. 2014). Furthermore, calculations predict that mRNA abundance and half-life values are compatible with the translocation rates of the transcripts in question. This led to the conclusion that sieve tubes do not discriminate between phloem-mobile macromolecules (Calderwood et al. 2016).

In cases of nonselective release via PPUs, the distribution of macromolecular signals strongly relies on the presence of *cis*-acting sequence elements, named "zip codes" or "tags" (Lucas et al. 2001; Aoki et al. 2005). Proteins that are necessary for local turnover of sucrose carriers such as SUT1 (Kühn et al. 1997) should be tagged for local use. Protein turnover in sieve tubes (Fisher et al. 1992), which requires back-flow of proteins into companion cells along the pathway, also requires tagging for bidirectional transport. Macromolecules arriving in the sinks should be tagged for specific destinations (Foster et al. 2002; van Bel et al. 2011). In conclusion, different zip codes could earmark macromolecules for a local destination in the adjacent sieve elements, for retrieval by companion cells along the pathways or for specific or nonspecific delivery into sink cells (Foster et al. 2002; van Bel et al. 2011). Despite the feasibility of a nonselective mode of macromolecular delivery via PPUs and a selective distribution of long-distance signals, plausible objections to stress-related release have again been raised against the concept (Schulz 2017).

## 11.9 Epilogue

In spite of the unmistakable progress made during previous decades, countless questions remain to be answered regarding the structure, diversity, and function of PDs, some of which are touched on in recent publications (e.g., Patrick et al. 2015). PDs seem to be highly specialized, given the diversity of PD structure and function. The associated structural and functional variety between PDs may explain why universal structural components of PDs have not been identified with certainty, despite many comprehensive attempts. Moreover, the gating mechanisms and their regulation are still unresolved, as well as the nature and regulation of the polar or bidirectional transport that seems to be necessary at some cellular interfaces. It also remains largely unclear if and how macromolecular trafficking is energized, how its selectivity is achieved, and how the cytoskeleton is involved in macromolecular passage. Related questions pertain to the selectivity of macromolecular PPU trafficking and the presence of target sequences (zip codes) attached to the macromolecular long-distance signals.

In addition to insights into the spatial arrangement of the cytoskeleton, we seem to have made considerable progress in understanding the molecular architecture of PDs. The idea may take root that we are close to deciphering PD structure and composition because we hold many pieces of the jig-saw puzzle. This point of view is attractive, but perhaps illusive. It is possible that we have collected pieces of diverse puzzles in one box. PDs may be different at one cell wall, or at the different walls of one cell; they are surely different in structure between various cell types with different functions, given the enormous morphological variety. This suggests a wide diversity in PD architecture and composition; Herculean efforts are needed to assess the molecular equipment of each single PD type.

Another major point of interest is the function of PDs in intercellular and interorgan communication as part of a larger framework. Symplasmic domains are dynamic units, the sizes of which are modulated in response to environmental and endogenous changes. How the dynamics of symplasmic domains are regulated and orchestrated during embryonic, juvenile, and mature stages of plant growth is largely unknown; neither is it understood how macromolecular, hormonal, and electrical messages are involved in the dynamic segregation and coupling of cell clusters at both local and systemic levels.

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# Chapter 12
# Origins of the Cellular Biosphere


**Jana Švorcová, Anton Markoš, and Pranab Das**

> *Sparse life hanging on in oases could never have the power to regulate or oppose the unfavorable changes that are inevitable on a lifeless planet. Sparse life would only be found at the birth or death of the Gaian system.*
> Lovelock 1988, 76.

**Abstract** Our basic axiom is "Life is a semiotic category," that is, living beings are able to decipher signs and grasp their meaning, and to behave according to their memory, experience, and momentary context. Semiosis requires memory and experience of both individuals, lineages, and the whole biosphere. First, we demonstrate this by developing on the concept of "umwelt," by understanding it as historical memory and as the experience of individuals or their assemblages (i.e., not only what is manifested here and now). This leads us to the idea of mutual understanding (to some extent) of all life in the biosphere. We discuss the means of message transmission in terms of both the intracellular protein ecosystem and the biospheric web, as well as the mutual influences of such systems. Hence, evolution of life and its biospheric web is rooted in universal protocols maintained by the mutual efforts of all biosphere dwellers.

## 12.1 The State of Art

The semiotic capacity of life depends upon the fact that neither living beings nor their communities arise de novo: they are born from parents into an already existing community that, in turn, also lacks a clear beginning (single-species ecosystems thriving in some extreme habitats are very rare). In this respect, species,

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populations, and ecosystems share many properties with human cultures and can be assessed analogically. All living beings ("autonomous agents," see Kauffman 2000) propagate the overall organization of the biosphere by co-constructing it, while being constituted by it. They experience their environment and subsequently interpret the inputs received so as to select a method of entry into the "adjacent possible." All such cues and actions are interpreted according to the memory and experience of the lineage,[1] thus expanding the realm of the agent's heritable potential.

Biosemioticians often adopt the term "umwelt" as coined by J. von Uexküll in order to stress the inner world of animals (von Uexküll 2010). Our aim here is to broaden the concept to acommodate extant development in evolutionary biology and epigenetics and to apply it not only to animals but to all of life; for a broader context, see Markoš (2014, 2016) and Markoš and Das (2016).

Uexküll sees umwelt as a miniature world tuned to the needs of the individual, but also walling off the rest of the world from exploration and involvement: "All that a subject perceives becomes his perceptual world and all that he does as a reaction to it, his effector world. Perceptual and effector worlds together form a *closed* unit, the Umwelt" (von Uexküll 1992, 320, italics ours). The whole universe, which consists of nothing but "autistic" umwelten, is held together by functional cycles/circles and unified according to a total plan to a single unit that we call Nature (Tønnessen et al. 2016).

Most definitions of umwelt share the Uexkullian static view of a perfect, immutable organization of Nature, and to his hostility toward evolution, especially its Darwinian version that does not allow for planning. An organism in its umwelt is instead a well-tuned, programmed machine or a robot.

Here we offer a concept of umwelt that is more dynamic (semiotic), taking into account the ever-evolving relation of a living being with its world. Such a relation is based on the interplay of stored genetic and epigenetic memory, experience and habits within the given lineage, and the context of community into which the living being is born. The community, in turn, is also characterized by an analogical interplay; it also develops the frame of its umwelt, a frame made malleable by the living agencies it consists of. Last but not least, physical parameters (climate, season, etc.) and natural selection may play a decisive, but not exclusive, role: there is always room for inner activity. All such factors reciprocally contribute to the state-of-the art umwelt.

To provide the reader with an idea of our approach, in Fig. 12.1 we provide schemes of the different understandings of umwelten. In Fig. 12.1a, we see the Uexküllian fixed plane of existence. In contemporary terms, the perceptual territory of a being is determined by a finely tuned execution of installed programs, triggered by environmental signals. Uexküll provides a charming parable: "The countless Umwelten represent the keyboard upon which nature plays its symphony of

[1]"Lineage" is the term used here for simplicity. As shown below, we are aware of the reticulate character of the "tree of life," as well as the fact that most evolution is evolution of holobionts.

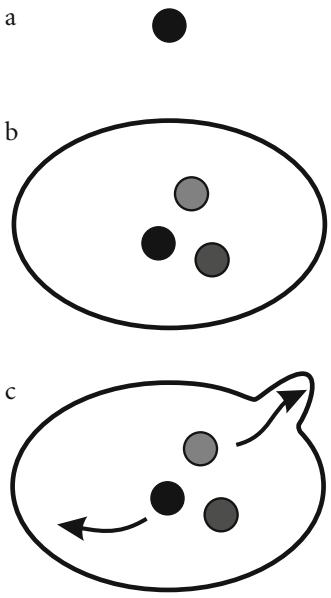


**Fig. 12.1** Views of the umwelt. (**a**) The circle represents the closed Uexküllian model of umwelt, a glockenspiel endlessly repeating the same melody. (**b**) Umwelt is represented by the outlines of the ring; it delineates that part of the world that is the endowment (experience) of a given lineage. However, a small part of such a capacity is, or may become, accessible to a given cell/individual/population, portrayed by symbols that may correspond to an individual in its life-course, an ontogenetic stage of that individual, a form of population in a given biome, etc. Such particular umwelten are much more limited than the whole field of possibilities, but can "float" in confines of the oval. (**c**) Evolutionary trajectories within the state space of a given umwelt marked out by the experience of the lineage (left arrow), or claiming new space from the surroundings by inventing novelties (right arrow). The background beyond the ovals represents the "world as such," part of the cosmos inaccessible for a given entity

meaning, which is not constrained by space and time. In our lifetime and in our Umwelt we are given the task of constructing a key in nature's keyboard, over which an invisible hand glides" (von Uexküll 2010, 114). Charming as it is, this vision reduces living beings to cybernetic automata: it is difficult to see how the umwelten of human-made automata would differ from living creatures in such a world view. Emmeche (2001) gives an account of differences between animals and robots, but such differences wane when one considers, as we do, all living beings and not only animals. The scheme in Fig. 12.1b is a modified version of the same, taking into account different "tuning" of the umwelt as a result of individual variations, ontogenetic stage, season, biospheric settings, etc.

This may correspond to what Tønnessen (2009, 2010) called the "transitional umwelt," defined as a "lasting, *systematic* change within the life cycle of a being, considered from an ontogenetic (individual), phylogenetic (population-, species-)

or cultural perspective, from one *typical* appearance of its Umwelt to another" (Tønnessen 2010, 383, our italics).[2] The change does not happen haphazardly, neither is dictated from outside, but is chosen. The author also holds, as we do, that an umwelt "necessarily refers to an underlying experienced world" (Tønnessen 2009, 61). Our views outlined in Fig. 12.1b-c resonate with his to a great extent, but we differ in that we seek to expand the concept to all life, not only animals as it appears Tønnessen does.

Figure 12.1b can also be read as a set of blots representing states into which the entire population might move under different circumstances or simply by virtue of contingency. In this case, the blots do not represent umwelten, but distinct states; the boundary circumscribes a set of all such conceivable umwelt states, utilized or not, or even not available under the reigning circumstances (environmental or evolutionary). This boundary maps out the entire historical experience, the endowment of a given lineage (species). In other worlds, what von Uexküll takes for an umwelt is but a single blot in a much broader state space representing the memory of experience of the lineage. Most of that space lies outside the umwelt of any given individual or population. New patches might be created under environmental stress (or, failing that, the population might terminate in extinction) but it is also possible that such stresses could be responded to by the recuperation of some former umwelt in frames of the state space.

There is a well-known and compelling case of such recuperation found in the stick insects (Phasmatodea; Whiting et al. 2003). This insect group evolved from ancestors who lost their wings tens of millions years ago. The time frame is important because conventional genetic theory supposes that such changes become irrecuperable over such long time frames. Surprisingly, stick insects can, in fact, recover their wings and do so independently in several extant lines in a way entirely consistent with the morphology of other winged insect groups. This strongly suggests that the wings are not an evolutionary novelty. Instead, they represent "remembering," a return to an umwelt within the penumbra of virtual umwelten associated not with a particular individual but with the entire life experience of that lineage.

A similar reversal can be seen in case of artificial induction in neotenic axolotl (*Ambystoma*), a development toward a terrestrial "adult" state that, in the contemporary biosphere, never appears under natural conditions. Quick adjustment of whole biota to abrupt climatic changes, (e.g., onset of a glacial period) may be rooted in a similar "remembering": After all, many glacial events took place in the past 2.5 million years. Such a latent historical experience is effectively the thesaurus of the whole community, extending much further than the areas in the state space occupied here and now.

[2]For multiple definitions of umwelt, see Tønnessen et al. (2016).

Evolution may also take a trajectory outward from within the realm of an existing state space, as shown in Fig.12.1c, to introduce not only recombinations and remembering based on ancestral experience, but also creating/incenting genuine novelties by expanding into the surrounding world, which was not available before. For example, the success of tetrapods was conditioned by the appearance of egg envelopes that enabled them to become independent on water basins. Similarly, land plants massively expanded after developing vascular tissues and after establishing mycorrhizal symbioses with fungi.

## 12.2 Symbioses: Overlaps of Umwelten

We understand the concept of "symbiosis" in its broadest sense, as cohabitation of living beings that come into mutual interaction of whatever quality such an interaction may be.

Promontories leaning out of the community's state space of umwelten (Fig. 12.1c) often lead to overlaps between two or more spaces of ecosystem dwellers that had developed in parallel, up to this point, without mutually influencing each other (Fig. 12.2). Although currently unconnected, they nonetheless share some common past because all forms of life sprang from common ground. Lineages evolving independently, often for billions of years, still share some common memory and experience with other lineages that are rooted either in a common heritage and/or in past encounters. Genetic and epigenetic codes, the workings and structures of cells and metabolic pathways are typical examples shared across the biosphere; they either reflect deep primeval organization or ways of conduct negotiated across the whole biosphere. However, *similar* does not mean *identical*; each lineage developed a slightly distinct "dialect" that may not fit other lineages. Hence, when lineages are brought into symbiotic bonds, great effort must be invested for such a union to be functional. They confront their previous understandings (environmental, developmental, nutritional, physiological) now in a partial overlap and may come to a mutual understanding that is in the fine-tuning, reorganizing, or inventing new usages within the repertoire of the umwelt. As the partners each reach outward toward one another, a jarring mutual reinterpretation, a consensus over such cues must take place. We might think of the resulting intercommunication as the emergence of a a sort of pidgin or creole language.

In the following sections we offer case studies as examples of symbioses interpreted as overlaps of two or more umwelten that came into a contact and established symbiotic communication.

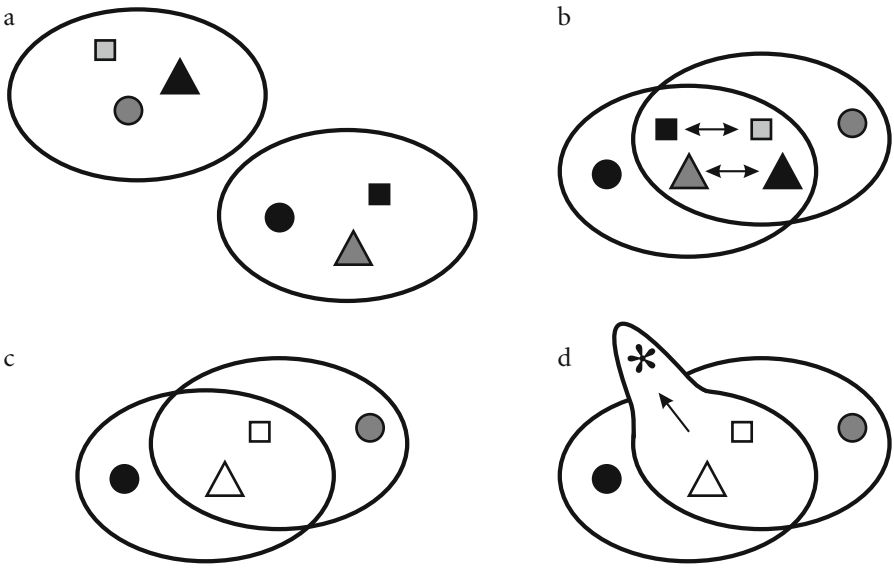


**Fig. 12.2** Symbiotic overlaps of different umwelten. As in Fig. 12.1, the background represents the world, ovals the state space, such as the potential of a given community and/or lineage. Patterns inside ovals do not correspond to particular umwelten but to different life strategies or features in a given state space. (**a**) Two single lineages, each with three features. Thanks to the common origin of both lineages, features are to some extent (square, circle) homologous, representing "dialects" of a common theme. (**b**) Two lineages come into contact. The overlapping area contains two similar but non-identical features of each partner, allowing mutual understanding to a certain degree. (**c**) "Symbiotic negotiating" leads to a state where homologous features assume common coordinates in the overlapping state space. (**d**) The symbiont claims a new realm in the surrounding world by reinterpreting some existing features or via a new invented feature (asterisk)

## 12.3 Biospheric Web

From its very beginning, the prokaryotic (Bacteria and Archaea) way of life has consisted of communities comprising tens to hundreds of different lineages of narrow specialization, united within a working consortia. The various tradeoffs required for sharing their lives within a web of elaborated ecosystems led to development of common network protocols to ease the mutual overlap. Such protocols embraced the evolution and maintainance of of a universal genetic code and of contraptions that allow extensive exchange of genetic material. This required the establishment of a sign system that result in a sophisticated symbolic (i.e., not metabolic) communication among dwellers of a given community, as in the case of sophisticated dynamic structures (such as mats, stromatolites, or floating fluffs) that enable and facilitate cell-to-cell contact and metabolic cooperation (for review, see O'Malley 2014). The interactions between species inhabiting such assemblies are so tight and unitary that they can be perceived as individuals, even from the perspective of natural selection (Ereshefsky and Pedroso 2013). Equipped with

such tools, prokaryotes conquered (or created) a plethora of niches (often very exotic) throughout sediments, water columns, and rocks. Most prokaryotes are not able to live axenically, without the presence of companions belonging to such a multicellular body (for a overview of recent progress, see Lok 2015). Such proficiency comes at a price: They very rarely build genuine multicellular bodies (i.e., the results of ontogeny, such as colonies; see 12.7).

Some eukaryotic lineages (especially unicellular protists) simply enriched the diversity of such microbial consortia. However, they also came with novelties that gradually transformed the face of the planet. Thanks to their genomes, relatively large size, sexual reproduction, and elaborated endomembrane system with exo- and endocytosis, they were capable of relative emancipation from the pervasive communication networks established by prokaryotes. Simultaneously, however, they added new dimensions to the exploitation of such networks.

With the emergence of sex, gene flow became restricted mainly to related individuals of the same lineage (a "vertical," i.e., parent-to-progeny gene flow), leaving less space for horizontal gene exchange with different lineages. This framing became a precondition for multiple (about 25) attempts towards multicellularity (Grosberg and Strathmann 2007), with the most sophisticated achievements in bilateral animals, vascular plants, and fruiting bodies in fungi. The structures of chromatin permitted cell and tissue differentiation of clonal material through the mutual influence (or manipulation) of and by cells in a common state space; the body can be taken as cell symbiosis of the closest possible kin. Moreover, ontogenesis became established along pathways marked more strictly than among "ecological successions" in bacterial consortia.

## 12.4 From Insulation Towards Holobiont

As a rule observed in many taxa, the earliest stages of ontogenesis must be thoroughly insulated from the surrounding ecologic web (Pátková et al. 2012). Early embryos develop in a sterile environment unimpeded by interference from other dwellers of the community. They receive their genetic endowment, as well as initial instructions and settings (e.g., egg cytoplasm containing a stock of nutrients, structures, and tools). Cell differentiation is correlated with epigenetic changes that may be stable throughout life, and even inherited. The newborn becomes finely adjusted to the conditions experienced by its progenitors, such as the lack of nutrients or new possible dangers (Markoš and Švorcová 2009; Gilbert and Epel 2015).

This splendid isolation of the newly formed individual is followed by a period of active coupling to the existing environmental web: the individual becomes coupled by manifold interactions with other dwellers of the biosphere (in viviparous animals, the descent through the birth canal or cloaca, ingestion of surface microbes on the teat, in pap, etc.). Such a sequence of events in multicellular development

ensures (1) relatively undisturbed conditions during the most critical phase of early ontogenesis; (2) sensitivity to maternal instructions; and (3) a relative freedom for the developing germ to establish functional and contextual links to the biospheric web (i.e., to become a "holobiont"; see Sect. 12.7). Some examples of the manifold ways of eukaryote cooperation (or, in our terminology, umwelt overlaps) are discussed in the next section.

## 12.5 Intracellular Symbioses (Endocytobiosis)

A eukaryotic cell may contain a host of prokaryotic or eukaryotic cells (in its cytoplasm, organelle, or other special structure), or derivatives thereof, with different degrees of autonomy of interacting symbionts. Such interactions are very rare among prokaryotes (see von Dohlen et al. 2001; Husnik et al. 2013); in contrast eukaryotes have become masters of such cooperation (or interdependence).

We next discuss symbiogenetic events whereby symbionts became organelles. Such symbiogenetic formation of organelles may seem to be extremely rare and mostly confined to the early biosphere. Yet, the contemporary biosphere has always been full of looser symbioses: there are fully fledged bacteria or archea living in the cytoplasm and often unable to thrive on their own; yet, such processes were never accomplished to the extent observed in mitochondria and plastids. As in the case of organelles, we witness a sophisticated coupling of both partners' metabolic and/or regulatory pathways, making them adjusted to a range of habitats (sediments, alimentary tracts, photic zones of water, etc.). When the host is "cured", for example with antibiotics, it often cannot survive because it is dependent on the metabolic products of the symbiont.

Protists (e.g., ciliates from the alimentary tract, or flagellates from the termite gut) host a broad selection of symbiotic prokaryotes in their cytoplasm. For example, the worker termite cannot digest cellulose without the help of its gut symbiont, *Mixotricha paradoxa*; the protist houses metanogenic archaea in its cytoplasm, and its surface is covered with bacteria (spirochaetae) engaged in the movement of the protistan cell (Wenzel et al. 2003). Altogether, the protist is a genetic composite of at least five other species (Gilbert et al. 2012). The same protist, in turn, inhabits the termite gut and, hence, represents part of the termite holobiont.

In symbioses with animals, another type of intracellular symbioses exists in thousands of variations, whereby only some tissues become infected. Thus, infection of the new generation must be ensured in the progeny: bacteria must leave their host cells and infect its progeny anew (e.g., Bright and Bulgheresi 2010). Exemplary in this respect are mycetomes, special animal tissues hosting intracellular bacteria (e.g. Baumann 2005). According to a rough estimate (Duron et al. 2008; Weinert et al. 2007), at least one third of arthropods rely on such maternally transmitted symbionts. Bacteria supply their hosts with essential nutrients and/or trigger their resistance to stress factors (Ghanim and Kontsedalov 2009) or parasites

(Vorburger et al. 2013). Bacterial presence may even influence the mating choices of their host, or other features of its phenotype (Feldhaar 2011).

Another example of such intracellular cooperation occurs in some animals dwelling at the interface between oxygenic and anoxygenic, euxinic marine zones. Sulfane ($H_2S$) is highly toxic for animals, yet it is an excellent fuel for many chemotrophic bacteria, which it provides with oxygen (Sievert and Vetriani 2012). Some animals, such as Pogonophora worms and bivalve molluscs, house such bacteria in their mycetomes. Such "chemotrophic" animals with atrophied alimentary tracts survive conditions at the boundary between anoxic and oxygen zones (e.g., deep sea hydrothermal vents) where no other animal can persist. Finally, we should mention intracellular photosynthetic symbionts (cyanobacteria or algae) inhabiting the cytoplasm of eukaryots, from protists to animals.

A fascinating case is the "serial endosymbiosis" in some lineages of photosynthetic eukaryotes: instead of capturing a prokaryote to serve as a chloroplast, they "enslaved" a eukaryotic alga and turned it into a "secondary" chloroplast (in Cryptophyta, Rhizaria, SAR, Excavate lineages). The story goes even further: some lineages might establish endosymbiosis with algae harboring secondary chloroplasts, and turn them as a whole into a tertiary chloroplast, a Russian doll of a kind, composed of what used to be three different eukaryotic and two bacterial lineages (Bhattacharya et al. 2003; Burki 2014). It should be mentioned that many now non-photosynthetic lineages still retain vestiges of secondary chloroplasts (Apicomplexa) or of a complete loss of them (e.g., Ciliata and Keeling 2010). Corals resemble animals with bacterial mycetomes: In this case, however, it is a eukaryotic alga, not a bacterium, that lives in the cytoplasm and provides the polyps with photosynthetic products. Finally, intracellular parasites, often causal agents of serious diseases, can be found in the realms of both bacteria (e.g., *Shigella*, *Salmonella*) and eukaryotes (e.g., *Plasmodium, Trypanosoma cruz*).

## 12.6 Cell-to-Cell Symbioses

The biosphere contains many tight symbioses between unrelated species, which often lead to very intimate interdigitation of both partners, yet each keep their cellular integrity intact. Bacterial communities were discussed above; here, we concentrate on examples of cohabitation between eukaryotes with other forms of life (eukaryotic or prokaryotic).

Plant roots often develop special structures that hold symbiotic bacteria. *Rhizobium*, a nitrogen-fixing, strictly anaerobic bacterium, receives shelter from oxygen in special nodules where it can thrive while exchanging nitrogen compounds with plant carbon assimilates (Masson-Boivin et al. 2009). A similar consortium of endophytic bacteria (bacteria living between cells inside the leaves) may perform the task in plant leaves (Knoth et al. 2014; Carrell and Frank 2014).

Mycorrhiza is a tight symbiosis of fungal hyphae with plant roots. Fungi are much better than plants at extracting nutrients from rock, which they supply to most land plants in exchange for organic compounds. The plant and fungal cells are very tightly interconnected, but again there is no merger of their cytoplasms. A classical example of such interconnection in lichens has been recently "upgraded" by findings that a lichen is, in fact, the symbiosis of three organisms: an alga and two different kinds of fungi (Spribille et al. 2016).

Note that mycorrhiza is not merely a dual relationship of a plant and a fungus. In fact, the mesh of fungal mycelia interconnects many plants. Moreover, the plant holobiont is not confined only to fungi: The roots organize the surrounding soil by secreting an extracellular scaffold of macromolecules, the structure is inhabited by many bacteria and protists, and the whole ensemble functions as a single organized unit – the rhizosphere (Berendsen et al. 2012).

Host–parasite symbioses very often reveal intricate information exchange that enables the parasite to become an integral part of the host, regulating (manipulating) its ontogenesis and functions, or even creating new organs. The gall induced by some insect larvae is effectively a special organ developed by a plant to provide shelter and nutrition to parasites. Another example is *Agrobacterium*, which has become a natural producer of genetically modified organisms. The bacteria, in root gall tumors, transform root cells with a special plasmid, enabling the plant to synthetize a unique class of organic compounds (opines) that serve as bacterial food (White and Winans 2007). Examples in animals include the cohabitation of squids (*Euprymna scolopes*) with a luminiscent bacterium, *Vibrio* (McFall-Ngai et al. 2012).

The strategy of protistan and animal parasites in animal tissues is even more sophisticated. Their evolutionary potential became oriented toward an intimate coupling with the host's regulatory cycles in order to avoid immune reactions and receive feeding. Examples include the crab parasite *Sacculina*, liver fluke (*Fasciola*), *Toxoplasma* (see Flegr and Markoš 2014), and blood trematodes (*Schistosoma*).

## 12.7 Symbioses in Ontogeny

"Holobiont" or genuine "biological individual" (Gilbert et al. 2012; Booth 2014) are names for an assembly comprising a eukaryotic "macroorganism" hosting an ecosystem of microbes or even multicellular organisms (Rohwer et al. 2002). Animal innards (guts and other cavities, skin, mycetomes) or plant rhizospheres serve as paradigmatic exemplars of such an assembly. In this section, however, we narrow our attention to the intracellular cohabitants of animals and to the mutual re-forming of participants of the holobiont game. Holobiontic assemblages develop their range and composition according to external cues or forces and/or ontogenetic stages.

It is tempting, of course, to include even the ecological interactions among holobionts in different biota, if not the whole biosphere-that-is-semiosphere, as

well as human affairs; however, we limit ourselves to cases where a "dominant" multicellular organism in the community can be recognized by its size, as in the case of the human body. Note also that a new multicellular organism enters its world in two phases: First, it is born to its mother (be it a single cell or a macroorganism); later it is born into its community.

By acknowledging that an "individual" organisms is actually an ecosystem, we also note that rarely do such individuals exist in the absence of their cohabitants. An axenic environment, preventing the developing organism from establishing metaholobiotic liaisons, often leads to a "germ-free" cripple. Its umwelt has become, in the course of evolution, tightly interconnected with those of other organisms and would collapse without them.

For example, the nutritional state of a macroorganism is generally dependent on the composition and well-being of its alimentary tract. Approximately $10^{14}$ bacteria reside in the human body, with the majority in the alimentary tract (over 1000 species, dominated by two bacterial phyla, Bacteroidetes and Firmicutes). Although the widely cited ratio of bacterial to human cells (10:1) has been questioned recently (Sender et al. 2016) and recalculated as being 1:1, the significance of bacteria for our health and development remains undisputable. They help us digest food, metabolize polysaccharides and cholesterol, and provide a plethora of essential vitamins, fatty acids, and other bioactive compounds (Nyholm and McFall-Ngai 2014). Drastic changes in microflora (caused, e.g., by antibiotics, pathogens, and allergic reactions, but also by gnotobiosis or the germ-free state) deeply change the ontogeny, functioning, and well-being of the macroorganism.

A holobiont is a result of an intricate ontogeny that involves all participants. We gain our first bacteria from our mother's vagina, mouth, and gut: "birth is nothing less than the passage from one set of symbiotic relationships [with mother] to another" (Gilbert 2014; see also Gilbert et al. 2012; Gilbert and Epel 2015). Some bacterial symbionts of the mother (e.g. bifidobacteria) are even able to overcome the placental and amniotic barrier and colonize the fetus before birth, helping development of its immune system and avoidance of pathogens. Bifidobacteria also serve as an elegant example of bacterial–human coevolution. Maternal milk not only supplies nutrients to the baby, it also supplies special oligosaccharides that are indigestible by the newborn but allotted as a nutrient for the first symbiont *Bifidobacterium longum bv. infantis*. The bacterium, in turn, produces short-chain fatty acids that are consumed by other bacteria that benefit the baby's gut and immune system (Sela et al. 2011).

Microbiota also influence the ontogenesis of the newborn, as can be demonstrated in germ-free laboratory animals that develop defective phenotypes. The increase in autoimmune diseases such as type I diabetes or multiple sclerosis can be influenced by disturbing some essential relationships with our inner microbial world (leading to dysbiosis), caused by excessive antibiotic use or an outlandish lifestyle (Lee and Mazmanian 2010). It has also been shown that mammalian symbiotic bacteria can influence the development of the nervous system and, consequently, behavior by producing different signaling molecules involved in

signal transmission and shaping of brain (Cryan and Dinan 2012; Forsythe and Kunze 2013).

## 12.8 Evolution

The Gaia theory (Lovelock 1988) argues that life can only exist as a planetary phenomenon, with all living forms tightly interconnected by uncounted mutual bounds—metabolic, structural, logistic, historical, and semiotic. Together with a well-documented genealogy of most lineages (Adl et al. 2012; Burki 2014; Plata et al. 2015; Nelson-Sathi et al. 2015), this vision delimits the stage for a hypothetical evolutionary scenario that highlights the various kinds of mutual encounters of beings belonging to otherwise distant branches of life (e.g. McFall-Ngai 2008). Whereas Lovelock takes into account physical-chemical interactions within planetary contexts, we propose that such interactions result from *mutual understanding* (of various degrees, of course), and root this in the presupposition that every member of the biosphere shares a common evolutionary origin. It is not the ambition of this paper to study the ultimate Gaian umwelt, we rather bring examples of lower-range umwelten. Here we will discuss the evolutionary aspect of inter-umwelt understanding.

In the introduction, we proposed to consider life as a semiotic category; this requires that life consists of systems which are born into a community and, so, have a history. 'Semiosis' in this usage automatically presupposes both 'birth into' and evolutionary (historically responsive) change: incessant process of interpretation and embedding of experience into the umwelt. The entire notion of being 'born into', the key point generally left unremarked upon in contemporary analyses, implies history and semiosis (Markoš 2014).

Of course, such a definition by no means excludes the dependence of life on mechanical parts and subunits. Biology has fruitfully explored cybernetic (code-controlled) and mechanical structures; but that rationalization has come largely at the expense of ignoring the semiotic processes requisite for life itself.

## 12.9 Conlusions: Evolutionary Ages from a Historical Perspective

### *12.9.1 Young Biosphere*

The classic textbook presentation of the common ancestor of life usually draws a picture of a prokaryotic-like creature that eventually (some 3.5 Gy ago) gave rise to Bacteria and Archaea, and much later, via multiple sequential mergers, to eukaryotic cells (2 Gy ago). A more contemporary perspective suggests instead that life proceeded from a cellular "mush"(a primordial "planetary holobiont") in which

cells promiscuously exchanged much more than mere genes (within a common biospheric genetic pool; David and Alm 2011). In the words of C. Woese: "The universal phylogenetic tree, therefore, is not an organismal tree at its base but gradually becomes one as its peripheral branches emerge" (Woese 1998). Cellular merger was also frequent, because there existed a common understanding among early inhabitants of the Earth. It is easy to understand this in terms of the umwelt. Because the state space available to these very simple organisms was small (phenotypic and experientially limited), because they occupied relatively undifferentiated subspaces in that overall space, and because these early forms shared much evolutionary history, their umwelten had strong overlap and so their capacity for mutual inter-recognition was very high.

This primordial "golden age" (Fig. 12.3, center) was followed by differentiation of the three domains known today. Archaea and Bacteria evolved toward a prokaryote-like lifestyle of "multispecies" consortia that were metabolically specialized yet interconnected via universal protocols of extensive horizontal gene transfer and pheromone signalization. The third domain, Eukarya, arose as a result of the multiple merger capacity of early cells. As a result of its relatively late appearance, the mitochondria is today's only easily recognizable evidence of such early blending processes, although many indirect clues point to earlier events.

The oldest fossils interpreted as remnants of eukaryotes are younger than 2 Gy; therefore, many researchers have taken the emergence of the eukaryote domain to be a rather late event. (The "eukaryote-early" hypothesis is offered by Glansdorff et al. 2008; for blending, see, Margulis 1993; Martin and Müller 1998; Mentel and Martin 2008; Woese 1998). We concentrate here on eukaryotes, tacitly presupposing that both prokaryotic domains adhere more or less to the lifestyles of their ancient precursors.

Eukarya are characterized by elaborated systems of internal membranes, mitosis and meiosis, and endocytosis and exocytosis; this last set of abilities includes the engulfing of or merger with other cells (fertilization is the most outstanding example). In exchange for information-processing abilities, they drastically reduced both their metabolic potential and interlineage gene flow. However, they retained and further developed their capacity for symbiotic cooperation with members of all three domains. Further evolution was framed within particular lineages (about 5–10 such supergroups, e.g., Burki 2014; Adl et al. 2012).

The age of the young biosphere was crowned, in one single eukaryotic lineage, by the symbiogenetic capture of cyanobacteria and their transformation to plastids (hence archiplastida). This algal lineage differentiated into rhodophyta and viridiplantae (plus the minor lineage of glaucophyta). This is perhaps the last instance of an overlap (understanding) of the umwelten of eukaryotic and prokaryotic cells to such an extent that they were able to become one cell.[3]

[3]The primary symbiogenetic event in *Paulinella* is capture of a cyanobacterium and its transformation into a primary chloroplast, some 50 million years ago. A single exception known from a rule suggested above.

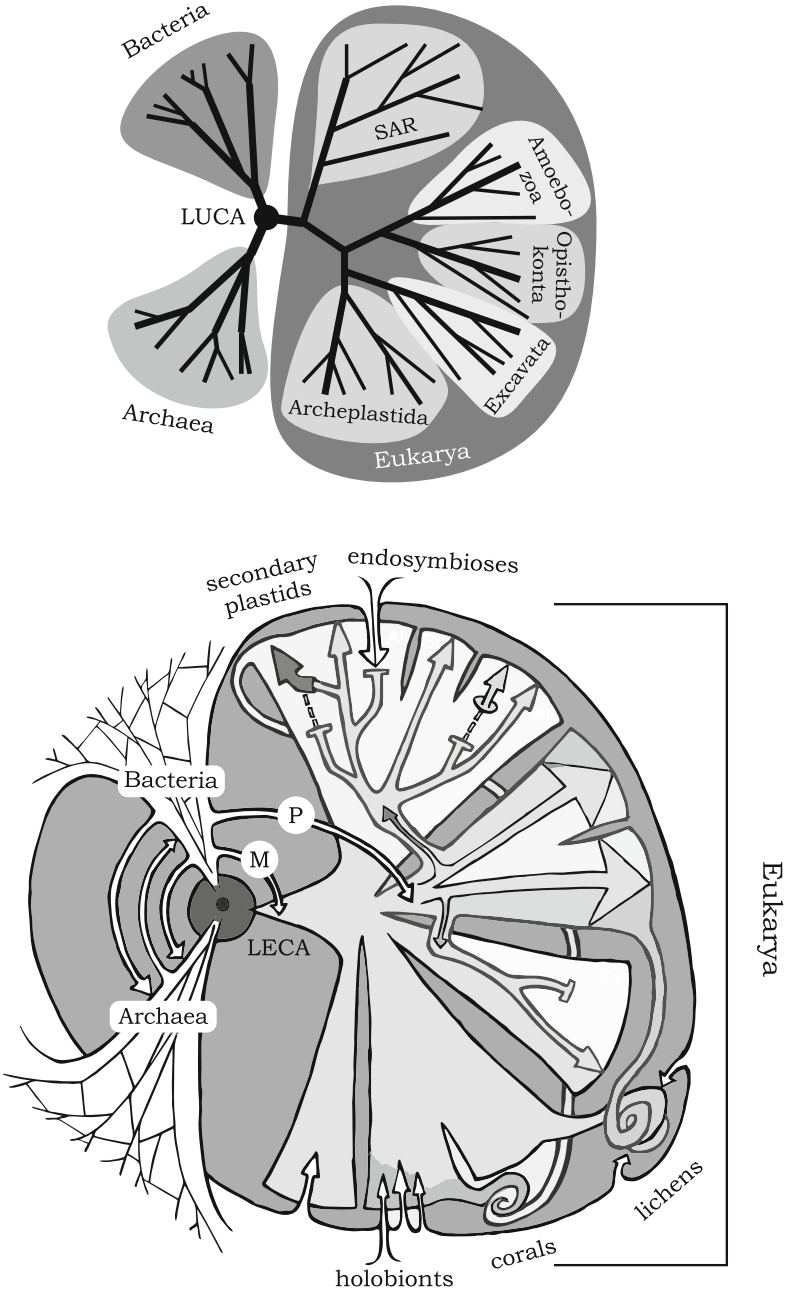


**Fig. 12.3** Symbiotic encounters in major evolutionary lineages. Above: The typical tree of autistic lineages, a tree of life typical ever since Darwin. Below: The scheme taking into account biospheric interactions. All three forms of life sprout from a biosphere of the last universal common ancestor (LUCA, at the center of the upper diagram), characterized by vigorous exchange of genetic material and merger of different cell types. The radial distance from that center very roughly corresponds to timeline (“ages”). Both prokaryotic forms of life (Bacteria and Archaea)

### *12.9.2 Middle Age*

In this stage of evolution, eukaryotes existed in easily recognizable (delimited) lineages. At first, they led lives that today would be called "protistan." They became tightly coupled members of multifarious consortia with prokaryotes and differentiated into a great number of lineages. Some established new kinds of symbiogenetic events, no longer with prokaryotes (as the intimate symbiogenetic understanding had been forgotten by this time) but with both red and green algae, as when a eukaryote turned into fully fledged organelle (a secondary chloroplast) within another eukaryote. In some lineages, evolution also proceeded by modification or loss of both mitochondria and chloroplasts (primary or secondary); Shiflett and Johnson 2010, and even by re-establishment of photosynthetic ability via a new capture of alga, in some cases establishing a tertiary symbiosis. Thanks to their size as well as their tendency to behave gregariously, protists also developed a plethora of tight holobiotic relationships with both prokaryotes and eukaryotes, which ranged from tight endocytobiosis to loose ecological relations in communities such as mats and plaques.

However, throughout this phase, the greatest invention of eukaryotes was the diversification of cell communities "from inside" by the creation of clonal multicellular bodies with well-defined ontogeny. We have seen that prokaryotes are also able to establish such bodies, but in their case it is an exception, whereas in multicellular eukaryotes it is a rule. About 25 parallel attempts towards multicellularity have been reported (Grosberg and Strathmann 2007), the most sophisticated achievement being reached in animals and green plants. Here, we witness perhaps the single example of "apobionts," wherein the developing germ and embryo are insulated from the rest of biosphere, coupled to it only through their parent(s). Avian eggs and mammalian fetuses are paradigmatic examples; insect eggs, with their load of mycetome bacteria are a counterexample. Even here, however, only

**Fig. 12.3** (continued) are characterized by extensive genetic exchange within the domain (reticulate evolution) as well as between both domains, and (less frequently) with eukaryotes. Eukarya resulted from multiple mergers of different cell types, followed by capture of α-proteobacterium, which turned into mitochondrion (LECA, last eukaryotic common ancestor). The lineage giving birth to plants also acquired a cyanobacterium that turned into a plastid. Eukaryotic lineages (thanks to sexual processes) keep tight genetic closure, allowing gene exchange almost exclusively only via sexual intercourse. Further symbioses led either to several endocytobiotic events leading to secondary and tertiary chloroplasts derived from algae, or to assemblages where partners can be easily distinguished, even if their cohabitation is very intimate and long lasting. Arrows inside the ring indicate the basic and most ancient symbiogenetic events: multiple genetic exchanges between Bacteria and Archaea, and capture of mitochondria (*M*) and plastids (*P*). Arrows connecting eukaryotic lineages point toward acquiring secondary (and tertiary) chloroplasts from the lineages of green or red algae. Kinks illustrate intimate symbioses, but not merger (e.g., animals with different protists, and plants with fungi). The outermost halo indicates the prokaryotic world, establishing a manifold of symbiotic events with Eukarya, some indicated by arrows. For more details, see text

the established symbiont is allowed to breach this rule. The germ soon establishes holobiotic liaisons with the life that surrounds it, with multiple overlapping umwelten.

### *12.9.3 The Age of Multicellular Organisms*

With the arrival of multicellular eukaryotes, the biosphere attained a new level of interaction (morphological, ecological, etc.) which does not fall within the scope of this paper. A holobiont can be flexibly "constructed" de novo according to its needs in the context of its surroundings. New contexts are recognized and life-styles attained not only by a random "insurance effect," as is supposed in bacteria (Boles et al. 2004), but by instruction from its kin community and its own endowment of memory (both genetic and epigenetic).

Interaction with other forms of life is either extracellular (e.g., mycorrhiza, gut microbiome) or intracellular, but confined to specialized cells (organs) and transmitted to a new generation in a controlled way. Hence, multicellular beings remain members of the biospheric web, yet jealously maintain their relatively emancipated state.

## 12.10 The Neo-Darwinian Versus Biosemiotic Interpretation

We offer a biosemiotic view of evolution with the ambition of grounding a view that parallels theories framed within the Neo-Darwinian synthesis. The issue is not the rather militant vocabulary of such theories, which abounds with enslavement, competition, cheaters, defectors, spite, conflict mediators, unicellular bottleneck as avoidance of parasites, allorecognition for defense purposes, etc.; many reports map the ways of symbioses in the context of Neo-Darwinian theory, and illustrate our point (e.g., Gardner and West 2004; Gardner et al. 2004; Grosberg and Strathmann 2007; West et al. 2007). They recognize four types of interactions (mutualism, competition, altruism, and spite) and analyze their evolutionary importance. Despite the emotionally loaded words, it is clear that these players of the game do not actually play; they interact as programmed robots that do not "want" to compete, defend, or so forth. By introducing the semiotic dimension, we aim at understanding living beings as living (i.e., active) players of the biosphere/semiosphere. It is true that semiotics does not fall within the province of experimental science, and steps apart from biology as such. We may therefore risk being accused of vitalism or mysticism. Our goal, however, is modest: to show that knowledge gained by "humanities" such as history, linguistics, or semiotics may bring new light to our understanding of evolution and life. After all, the vernacular

meaning of words such as mutualism, etc., also points toward understanding. We strongly believe that concepts such as "basal level of description," "upward/downward causation," "higher/lower level of organization" will not bring us to a complete understanding of complex dynamic systems (Švorcová 2016). This is particularly true for systems that are born (i.e., do not pop up de novo). Recognizable agencies in living beings (or societies of living beings) are equipollent. They influence each other and evolve in contribution to each other's development (hence, "reciprocating" and "re-forming"). In complex dynamic systems, many such mutually influencing agencies can be recognized and each can enter the focus of the observer's exploratory perspective. The principal presupposition is that all living beings share at least a small part of their experience, that which is inherited from common ancestors. In their mutual encounters within ecosystems, holobionts, host–symbiont relationships, etc., living beings take advantage of the knowledge of their partner(s) and develop a wide array of games. Human language and culture may serve as a suitable analogy for these situations.

An overlap involves exploratory activity from both perspectives in order to decipher the meaning of common elements (words, signaling pathways, or behavior) that look the same or similar, yet may be "wired" differently in the partner, and attached to different meanings. This is because, although signal-triggers and even complete signaling pathways may be shared, each partner arrives at a different interpretation (which they then take into new encounters). The overlapping of umwelten may end in a different degree of symbiosis, antagonism, or conflict (and the potential destruction of one partner), or to a new separation.

It is important to appreciate that this is the process of mutual enculturation. The fruit of our vision is found not only in phenotypic similarities within ecological niches, but also in a whole set of processes by which one understands and dwells within our shared world. These processes are not of genetic coding or in phenotypic expression, but part of the long-term, ongoing experience of the world as manifested by and within a living being. Both this experience and the actual meanings of things differs with the community of life. Cohabitation within the biosphere, which we describe as the overlapping of umwelten, requires a deciphering of meaning that, in turn, depends on a "knowledge" (capacity to process) of context and semiotic analogies, which come into view with the overlap (see e.g. the concept of semetic rings; Kleisner and Markoš 2005). Such an interpretation may or may not be successful; individuals as well as communities may resist understanding, they may misinterpret, paraphrase, re-interpret, forget, or escape the conversation by inventing novelties.

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