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Cytoskeleton functions in plant–microbe interactions

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Abstract

The plant cytoskeleton consists of actin filaments and microtubules and plays a pivotal role as mediator of intracellular transport processes. In addition to this essential cellular housekeeping task it significantly contributes to the establishment of cell polarity during plant development and morphogenesis. Rapid changes in regular cytoskeleton architecture occur upon contact of individual plant cells with both pathogenic and symbiotic microbes. In the case of pathogens, polarized cytoskeletal rearrangements are thought to allow the localized delivery of cargo for defense execution, while in symbiotic interactions the reorganization may advance establishment of the symbiotic relationship. Although firm experimental evidence for these facts is lacking to date, it is believed that microbial metabolites and effector proteins are released into plant cells for manipulation of the host cytoskeleton, while some secreted plant defensive polypeptides may target the microbial cytoskeleton. This would be consistent with the recent finding that cytoskeletal functions are not only crucial for plant defense but likewise essential during various stages of microbial pathogenesis. The cytoskeleton thus emerges as a potential mutual target in plant–pathogen combats that appears to be under attack by effector molecules from both sides.
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Keywords: Arbuscular mycorrhiza; Arbuscules; Rhizobia; Infection thread; Host cell entry; Haustorium; Non-host resistance; Race-specific resistance; Prepenetration apparatus; Tip growth; Spitzenkörper

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Abbreviations: ADF, actin depolymerizing factor; AM, arbuscular mycorrhiza; GFP, green fluorescent protein; HR, hypersensitive response; IT, infection thread; NF, Nod factor; PPA, prepenetration apparatus.

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1. Components of the plant cytoskeleton

Plant cells harbor a complex and highly dynamic three-dimensional scaffold comprised of filamentous polymers (actin filaments and microtubules), that is collectively referred to as the cytoskeleton. Actin filaments consist of two intertwined actin chains (each representing a polymer of actin monomers) whereas microtubules are hollow supramolecular structures composed of protofilaments. Each protofilament in turn represents a polymer of α and β-tubulin subunits. Thus, monomeric globular actin (G-actin) molecules as well as α and β-tubulin subunits form the basic building blocks of the cytoskeleton. Actin is a highly conserved structural 42 kDa protein that can be assembled into long filamentous polymers (F-actin) in a directional fashion. Two parallel F-actin strands usually form higher order structures, the so-called microfilaments. These may further congregate in densely packed actin cables which are assumed to be the major tracks for intracellular organelle trafficking. Microtubules are polymers of α- and β-tubulin dimers that directionally polymerize end-to-end into protofilaments. An assembly of 13 protofilaments forms the hollow and cylindrical filaments (microtubules) that represent the basic structures of the microtubular network. Owing to the intrinsic polarity of the basic subunits they are composed of, both actin filaments and microtubules have a defined polarity. In both cases the so-called plus end is more dynamic and has a higher subunit turnover than the minus end (for a comprehensive portrayal of the plant cytoskeleton refer to [1]).

A plethora of accessory proteins assist and regulate the formation and organization of cytoskeletal polymers and control their assembly in space and time. More precisely, these polypeptides determine the site of polymer nucleation, the direction and speed of polymerization and the potential branching of actin filaments. They further govern the stabilization of existing polymers, their organization in higher order structures, the fragmentation of unnecessary cytoskeletal elements and the attachment of polymers to other cellular structures. Actin binding proteins (ABPs) regulate the remodeling of the actin cytoskeleton by regulating actin filament assembly and disassembly (reviewed in [2]). Prominent members comprise actin depolymerizing factors (ADFs; also known as cofilins), profilins, formins, villins, fimbrins as well as subunits of the Arp2/3 complex. In particular, profilins and ADFs, which polymerize and depolymerize actin filaments, respectively, are major mediators of actin dynamics and architecture [3]. Regulators of the microtubular network include members of the MAP65 family, SPC98, γ-tubulin, MOR1/MAP215, EB1, phospholipase D (PLD), SPR1/SKU6, SPR2/TOR1 and katanin p60 [4,5]. Although most of the biochemical activities of these proteins can principally be inferred from their animal and yeast counterparts, the cellular functions of these polypeptides have only been vaguely unraveled so far in plants. At least some of the regulatory polypeptides, e.g. the Arp2/3 complex, have acquired plant-specific roles [6], while further as yet undiscovered proteins may fulfill additional tasks in plant cytoskeletal organization and turnover.

Besides proteins that directly bind to cytoskeletal mono- and polymers, a range of regulatory polypeptides indirectly influence cytoskeletal assembly. Possibly the most prominent members of this class are small guanine–nucleotide-binding and -hydrolyzing proteins (GTPases) of the Rac/Rho family [7] that regulate actin architecture. It is thought that signal transduction from Rho-like GTPases of plants (ROPs) to the cytoskeleton occurs via ROP-interacting Cdc42/Rac interactive binding (CRIB) motif-containing proteins (RICs; [2]). Another class of polypeptides that indirectly shape the actin cytoskeleton are calcium-dependent kinases (CDPKs). CDPKs represent a class of calcium-stimulated serine/threonine kinases that are only present in plants and some protists. At least some CDPKs seem to phosphorylate and by this means inactivate ADFs, thereby indirectly modulating actin filament turnover [8].

2. Cytoskeleton functions in plants

The roles of the cytoskeleton in plant biology are multifaceted. At the cellular level, it provides the major tracks for intracellular transport. Several plant organelles, e.g. Golgi stacks [9], peroxisomes [10] and mitochondria [11], have been shown to be shuttled along actin filaments/cables as part of the active and continuous mass movement of organelles in the cytoplasm that is referred to as "cytoplasmic streaming". Myosins represent the molecular motor proteins that provide the driving force for organelle traffic along actin filaments in an energy-dependent manner [12]. In analogy to the situation in yeast and mammals, intracellular transport of membranous vesicles between the components of the plant endomembrane system is also thought to be mediated by the concerted activity of actin filaments and myosin motors [12,13].

Besides these basic "housekeeping" roles in vegetative, non-dividing interphase cells, cytoskeletal polymers have additional crucial functions in dividing cells. Although it appears that actin filaments are dispensable for the early stages of mitosis they are essential for formation of the cell plate during cytokinesis, the separation of daughter cells after the successful segregation of chromosomes into the daughter nuclei [14]. Actin filaments further modulate stomatal opening in guard cells [15] and likely establish connections to the cell–cell conduits called plasmodesmata [16]. The dynamic reorganization of actin filaments is essential for the transduction of gravitropic stimuli in root cells [17] and represents a recurrent theme in plant–microbe interactions (reviewed in [18]; see below). Microtubules, on the other hand, organize the positioning of multiprotein cellulose synthase complexes at the plasma membrane, thus determining the site of primary cell wall biosynthesis [19]. Additionally, microtubular arrays fulfill various roles

throughout the plant cell cycle, e.g. in chromosome segregation and cell wall positioning during mitosis [20]. One of the currently unresolved questions is how plant cells regulate microtubular organization in the absence of a conspicuous microtubule organizing center (MTOC) such as the centrosome, the cellular structure that nucleates microtubules in other eukaryotes [21]. It has been recently proposed that soluble γ-tubulin-containing complexes permit nucleation of microtubules at dispersed sites in plant cells [22].

Tip growth is an extreme form of polarized growth of single living cells that results in an elongated cylindrical cell morphology with a rounded tip at which the growth activity takes place. In plants, tip growth occurs in root hairs and pollen tubes. It relies on the focal assembly of exocytotic vesicles at the apices of growing tips and has been shown to depend on actin filaments, while microtubules seem mostly dispensable for this process [23,24]. Although largely reminiscent of tip growth in pollen tubes and root hairs, morphogenesis of unicellular, yet branched, leaf trichomes in *Arabidopsis thaliana* is more complex and possibly involves more than one growth mechanism [25]. Nevertheless, trichome morphogenesis depends on organized filamentous actin [25], while trichome branching might be associated with transiently stabilized microtubular structures [26]. In sum, the cytoskeleton fulfills versatile tasks in plant life, of which many are associated with the establishment and/or maintenance of cellular polarity.

3. Cytoskeletal rearrangements in plant–pathogen interactions

The first descriptive studies of the cytoskeleton in the context of plant defense date back to the early 1990s and were driven by the startling observation of extensive host cell polarization upon pathogen attack (reviewed in [27]). In challenged plant cells, cytoplasmic aggregation is commonly observed below the attempted microbial entry site. This often coincides with migration of the nucleus towards the site of invasion [28,29]. Kobayashi and coworkers used immunocytochemistry to study microtubule organization in barley coleoptile cells attacked by the non-host pea powdery mildew pathogen, *Erysiphe pisi*. They found that in non-challenged cells, microtubules formed a transversal array along the longitudinal axis of the cells, while they gathered as a radial network at attempted fungal entry sites [30,31]. Similarly, rhodamine–phalloidin staining coupled with confocal laser scanning microscopy revealed thick actin cables and fine microfilaments which were arranged longitudinally in healthy barley coleoptile cells. Following inoculation with *E. pisi*, redistribution of actin filaments towards the attack site occurred, thereby establishing a radial network focused towards the incipient fungal entry site [31,32]. Using the same techniques, similar arrangements and redistributions of microtubules and actin filaments towards sites of fungal or oomycete attack were observed in a range of compatible plant–microbe encounters, including the flax-*Melampsora lini* [33], soybean—*Phytophthora sojae* [34], onion—*Botrytis allii* [35] and barley—*Blumeria graminis* f.sp *hordei* [29] interactions (Fig. 1). Consistent with these findings, the local accumulation of modifiers of cytoskeletal organization such as profilin and ROP GTPases has been reported to take place at plant–oomycete infection sites [36]. Taken together, focal orientation of the cytoskeleton in the direction of biotic stress sites occurs in a broad spectrum of plant species and cell types and upon attack with different classes of pathogens that have diverse lifestyles (fungi and oomycetes, biotrophs and necrotrophs). These features suggest that this phenomenon is a widespread and conserved cellular response contributing to plant defense.

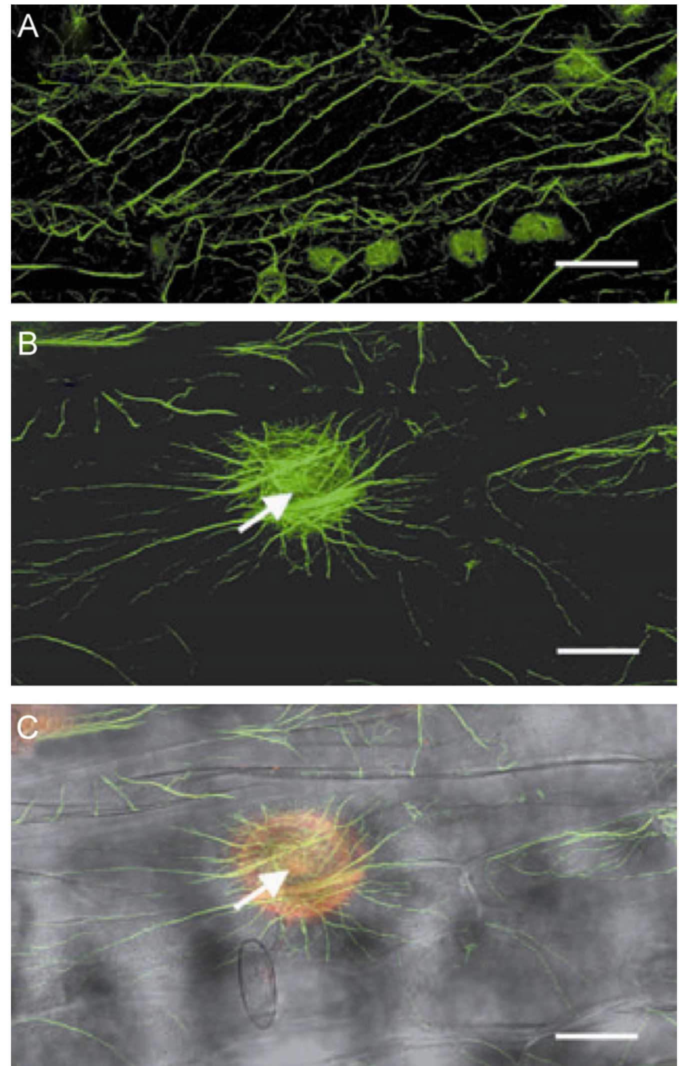

Fig. 1. Polarization of actin filaments towards abiotic stress site. Confocal images of Alexa Fluor 488 phalloidin-stained actin filaments in a barley leaf epidermal cell before (A) and after (B, C) attack by a powdery mildew sporeling. (A) Actin bundles (shown in green) are oriented transversally to the longitudinal cell axis. (B) The same cell as shown in (A) visualized at 18 h post-inoculation with a spore of *Blumeria graminis* f.sp. *hordei*. The arrow points to the actual attack site. (C) Overlay projection of two confocal channels and the transmission channel. Bars, 20 μm (reproduced with kind permission from [29]).

It is even induced by a non-pathogenic *mps1* mutant strain of the rice blast fungus, *Magnaporthe grisea*. The *mps1* mutant is arrested in the stage of appressorium formation and, despite the presence of a penetration peg, unable to access host cells [37]. It is, however, not unequivocally resolved whether the perception of fungal elicitors or mechanical stress trigger cytoskeletal rearrangements since another apothogenic *M. grisea* mutant that is also arrested at the appressorial stage but does not form a penetration peg (*mst12*) does not provoke any alterations in host cytoskeleton organization [38].

Transient expression experiments in barley leaf epidermal cells indicate that the focal reorganization of actin filaments towards powdery mildew attack sites is controlled by a member of the ROP family, RACB [29]. Gene silencing and overexpression of a constitutive active protein variant revealed that the barley RACB GTPase affects the degree of powdery mildew entry into host cells [39–41]. Together, these findings correlate polarized cytoskeletal reorganization with the success rate of antifungal defense at the cell periphery. Likewise, cytoskeletal rearrangements of both microtubules and actin filaments occurred less frequent and markedly decelerated in interactions of barley coleoptile cells with the compatible powdery mildew pathogen, *Blumeria* (formerly: *Erysiphe*) *graminis*, compared to the interaction with a non-pathogenic powdery mildew fungus [32]. This observation and similar findings in other pathosystems (e.g. [29,33]), further support the hypothesis that the plant cytoskeleton has a role in defense and additionally suggest that adapted pathogens might interfere with these cytoskeletal rearrangements, possibly by release of effector proteins that compromise cytoskeleton functions (see also below). It should be noted, however, that in some pathosystems no obvious correlation between the extent of actin reorganization and disease resistance could be observed [28,42]. In addition to an altered cytoskeletal pattern, actin monoubiquitination has been reported to be induced in pathogenic and symbiotic plant–microbe interactions. The post-translational modification with solitary ubiquitin molecules was predominantly found in filamentous, membrane-associated actin and was hypothesized to provide increased stability against proteolytic degradation [43].

The results of the above-mentioned studies were obtained by visualization of cytoskeletal polymers via either immunocytochemistry (microtubules) or rhodamine–phalloidin (actin filaments) staining. These techniques require prior fixation of the sample tissue, a procedure that is tedious and may possibly generate artifacts. Recently, the development of genetically encoded fluorescent probes that decorate cytoskeletal components alleviated the experimental hurdles to display cytoskeletal architecture and allows visualization of cytoskeletal dynamics *in planta* and in real-time [4,44,45]. Despite some inherent limitations [4], these probes offer the exciting advantage of using living cells, thus avoiding the need for tissue fixation. Studies employing such fluorescent reporters essentially corroborated the above-mentioned patterns of the plant cytoskeleton in resting and its focal reorganization towards the potential site of invasion in attacked cells [42,46–49].

4. Pharmacological interference with host cytoskeletal functions in plant–pathogen interactions

Experiments involving pharmacological inhibitors of cytoskeleton integrity provided first convincing evidence for an authentic role of the cytoskeleton in plant defense. Cytochalasin and phalloidin are compounds that prevent actin filament polymerization and de-polymerization, respectively (Table 1). Likewise, colchicine, taxol, oryzalin and propyzamide affect microtubule architecture (Table 1). Treatment with various types of cytochalasins allowed successful entry (indicated by the formation of functional fungal haustoria) by the non-adapted pea powdery mildew pathogen, *E. pisi*, into barley and wheat coleoptile cells as well as into epidermal cells of tobacco and cucumber. Moreover, cytochalasin E application enabled successful entry of a range of further non-adapted fungi, such as *B. graminis* f.sp. *hordei*, *B. graminis* f. sp. *tritici*, *Sphaerotheca fuliginea*, *Colletotrichum graminicola*, *Colletotrichum lagenarium*, *Mycosphaerella pinodes*, *Alternaria kikuchiana* and *Corynespora melonis* into both barley coleoptile and tobacco leaf epidermal cells [50]. Semiquantitative assessment of the degree of actin (de-)polymerization revealed an inverse dose-dependent correlation between the level of polymerized actin and the penetration efficiency of *C. graminicola* and *E. pisi* into barley and wheat coleoptile cells [31,50]. In comparison to drugs interfering with microtubules (i.e. oryzalin, colchicine and taxol), the actin polymerization inhibitors cytochalasin A and E had a much stronger effect in conferring successful entry by *E. pisi* into barley non-host coleoptile cells [31]. Co-application of compounds interfering with both microtubules and actin filaments showed an additive but not a synergistic effect on the fungal penetration rate [31]. Taken together, these observations suggest that actin filaments play a major and microtubules a minor role in penetration resistance of mono- and dicotyledonous plants against non-adapted powdery mildew fungi. Notably, these data also indicate that plant actin cytoskeleton function appears dispensable for the establishment of fungal and oomycete feeding organs (haustoria) inside successfully penetrated plant cells.

The pivotal effect of actin filaments in penetration resistance has subsequently been demonstrated in a range of further plant–microbe encounters, such as the interaction between barley and the rice blast pathogen, *M. grisea* [51], the interaction between *A. thaliana* and non-adapted powdery mildew [52] or *Colletotrichum* species [48], and the interaction between barley and the adapted powdery mildew pathogen, *B. graminis* f.sp. *hordei* [53]. Intriguingly, the effect of cytochalasins on penetration resistance against non-adapted powdery mildews has been shown to be even maintained in dedifferentiated cultured tobacco

Table 1
A selection of drugs commonly used for pharmacological interference with cytoskeleton function in the context of plant-microbe interactions

Effect on cytoskeletal polymer	Drug name	Origin	Substance class	Effect on cytoskeleton	Reference	Examples of usage in the context of plant–microbe interactions
Actin filaments	Cytochalasins	Fungal metabolites (mycotoxin)	Alkaloid	Binds to barbed end of actin filaments, blocks assembly and disassembly	[125]	[28,31,48,50,51,53,54,57,60,62,94]
	Phalloidin	Poisonous mushrooms (*Amanita phalloides*)	Bicyclic heptapeptides	Preferentially binds at the interface between F-actin subunits, locking adjacent subunits together, preventing depolymerization	[125]	[31]
	Latrunculin	Certain sponges (e.g. genus *Latrunculia*)	Natural product	Binds actin monomers near the nucleotide binding cleft with 1:1 stoichiometry and prevents them from polymerizing	[126]	[113]
microtubules	Oryzalin® (3,5-dinitro-N4, N4-dipropylsulfanilamide)	Synthetic herbicide	Dinitroaniline sulfonamide herbicide	Binds to plant tubulin and inhibits microtubule polymerization *in vitro*	[127]	[31,53,94]
	Paclitaxel (Taxol®)	secondary plant product from the bark of trees of the genus *Taxus*	taxane diterpenoid (taxoid)	stabilizes microtubular structure by binding to a pocket of β-tubulin on the microtubule's inner surface	[128]	[28,31]
	Propyzamide®	Synthetic herbicide	Substituted benzamide; amide herbicide	Inhibits microtubule polymerization	[129]	[53]
	Colchicine	Secondary plant product of the genus *Colchicum*	Tropolone alkaloid	Inhibits microtubule polymerization by binding to tubulin dimers	[130]	[31]
	Benomyl®	Synthetic fungicide	Benzimidazole fungicide	tubulin depolymerizing (selectively toxic to micro-organisms and to invertebrates	[131]	[38]

(*N. tabacum* BY-2) cells [54]. Collectively, these findings suggest that the reliance of host cell entry control on the actin cytoskeleton is widely conserved throughout the plant kingdom. Experiments employing pharmacological interference with actin cytoskeleton functions in Arabidopsis mutants defective in components of basal defense revealed that actin indeed primarily contributes to limiting host cell entry while the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) protein is a key player in post-invasive defense. In combination, loss of cytoskeletal and EDS1 function severely compromised non-host resistance of *A. thaliana* against the wheat powdery mildew pathogen, *B. graminis* f.sp. *tritici* (*Bgt*), and allowed completion of the asexual *Bgt* life cycle on *eds1* mutant plants [52]. These results are in accordance with the recently suggested organization of non-host plant immunity, proposing genetically separable layers of pre- and post-invasive defense [55].

5. Genetic interference with host cytoskeletal functions in plant–pathogen interactions

Although pharmacological interference generally represents an accepted and common approach to tackle the role of cytoskeleton function in plant–microbe interactions, it has at least two major shortcomings. As with all other pharmacological approaches, secondary effects of the compounds on cellular targets other than the one under investigation cannot be ruled out. Such side effects may directly or indirectly impinge on the experimental outcome, thereby possibly leading to wrong conclusions. Probably even more problematic is the potential effect of these drugs on the pathogenic intruder. As outlined below in further detail, the microbial cytoskeleton also plays an important role during plant colonization and is principally sensitive to anti-cytoskeletal drugs. Application of compounds that non-selectively compromise cytoskeleton functions may thus impede both the plant and the microorganism. A potential solution to this problem is the genetic interference with host cytoskeleton functions by (over-) expression of suitable proteins. Miklis and co-workers recently used transient overexpression of various ADFs inside barley leaf epidermal cells to probe the requirement of the host actin cytoskeleton in the context of powdery mildew attack. Overall, the authors found a good agreement between pharmacological and genetic interference with the actin cytoskeleton, suggesting that the genetic approach is a valuable complement to the experimental repertoire [53] (Fig. 2).

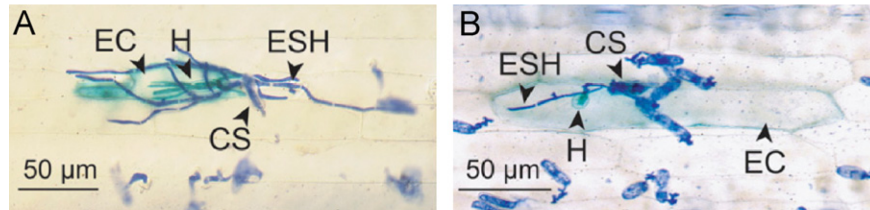

Fig. 2. Successful invasion of barley leaf epidermal cells upon genetic interference with actin cytoskeleton function. The micrographs show ballistically transformed cells (visualized by the greenish color of reporter gene expression) of the barley leaf epidermis overexpressing a barley *ADF* gene. Transformed cells have been successfully penetrated by sporelings of either the non-adapted wheat (*B. graminis* f.sp. *tritici*) (A) or pea (*Erysiphe pisi*) (B) powdery mildew pathogen. CS, conidiospore; EC, transformed epidermal cell; H, haustorium; ESH, elongating secondary hyphae (reproduced with kind permission from [53]. Copyright by the American Society of Plant Biologists).

6. The plant cytoskeleton is essential for various types of antimicrobial defense

The requirement of the plant cytoskeleton has been studied in the context of various types of plant defense, namely non-host resistance (interactions between plants and non-adapted pathogens; [31,48,50,52,53]), basal defense (interactions between plants and adapted pathogens; [53]), broad-spectrum powdery mildew resistance conditioned by barley *mlo* mutant alleles [53], and race-specific resistance [28,33,53,57]. The latter type of defense is often associated with localized cell death of the attacked cell, a phenomenon which is commonly known as the hypersensitive response (HR). In flax (*Linum usitatissimum*) cells attacked by incompatible races of the flax rust fungus, *Melampsora lini*, cytoskeletal changes prior to the execution of hypersensitive cell death were variable but consistently associated with a disappearance of the microtubular network [28,33]. Similarly, in tobacco, cell death triggered by application of the oomycete elicitor cryptogein strictly coincided with a rapid disintegration of the microtubules [58]. At least in one reported case, treatment with oryzalin delayed the HR to such an extent that an otherwise avirulent race of flax rust was able to form haustoria at a rate similar to that achieved by a virulent race in a compatible interaction [57]. Enhanced host cell entry was, however, not observed in the context of several tested race-specific barley-powdery mildew interactions upon application of drugs interfering with microtubule function [53]. While the relationship between microtubular fragmentation and the HR is still merely correlative to date, actin polymerization was repeatedly found to be required for the execution of HR-like cell death, since application of cytochalasins inhibited this response in several plant–microbe interactions [59,60]. Collectively, pharmacological and genetic interference with the cytoskeleton revealed a crucial role for actin filaments and microtubules in plant defense. In particular actin filaments appear to be instrumental for various types of defense responses.

7. Potential roles of the cytoskeleton in plant–pathogen interactions

What might be the reason for the impaired defense response upon interference with the plant cytoskeleton? Application of actin inhibitors in barley coleoptiles caused a dramatic decrease in the incidence of prototypical defense-associated cellular responses such as cytoplasmic aggregation, the formation of localized cell wall appositions (papillae) and the focal deposition of fluorescent material including the β1–3 glucan callose at incipient fungal entry sites [31]. In contrast, the reduction in these defense responses were significantly less pronounced upon application of drugs impeding microtubule activity, suggesting that actin filaments are primarily responsible for these subcellular rearrangements [31]. Work in other experimental systems corroborated the role of actin filaments in the directed translocation of cytoplasm and nuclei [47,61,62] and callose deposition [48,63]. In potato tuber disks, cytochalasin treatment led to a delay in the transcript accumulation of a defense-associated gene (phenylalanine ammonia lyase, PAL) and the accumulation of pathogenesis-related (PR) proteins upon treatment with *Phytophthora infestans* cell wall components [61]. In sum, it appears that the comprehensive reorganization of actin filaments is required for a number of cellular defense-associated responses, which may collectively contribute to arrest or restrict attempted invasion of plant cells by microbial intruders (reviewed in [64]). These responses likely involve the targeted delivery of defense-related cargo into the apoplastic space, e.g. antimicrobial low molecular weight compounds (so-called phytoalexins) and protective polypeptides (so-called pathogenesis-related proteins). This assumption is further supported by the fact that some SNARE proteins, decisive components of the plant secretory machinery mediating membrane fusion events , have been genetically determined to be required for successful defense in some cases [65–68]. Focal secretion of toxic cargo towards a foe, in association with rigorous cellular polarization based on comprehensive cytoskeletal reorganization, is mechanistically reminiscent of immunological synapse formation in mammalian cells [69]. Immunological synapses are the contact sites between specialized mammalian immune cells, the so-called cytotoxic T cells, and their infected or malignant target cells. Focal secretion thus appears to be a common theme in plants and animals to combat enemies at the cellular level (reviewed in [69]).

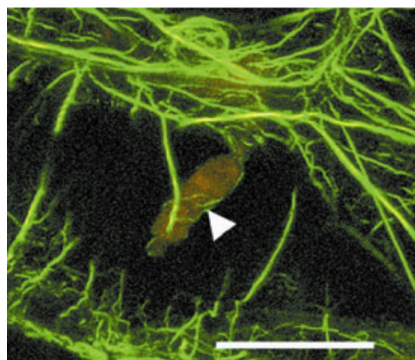

Fig. 3. Association of actin filaments with an emerging powdery mildew haustorium. Confocal images of Alexa Fluor 488 phalloidin-stained actin filaments in a barley leaf epidermal cell successfully penetrated by a powdery mildew (*B. graminis* f.sp. *hordei*) sporeling (image taken at 18 h post-inoculation). Green structures represent stained actin cables/filaments; the immature fungal haustorium is indicated by red autofluorescence (arrowhead). Bar, 20 μm (reproduced with kind permission from [29]).

In addition to filaments and cables that are polarized towards the attempted attack site before host cell penetration, actin filaments were also found to be tightly associated with haustorial complexes and biotrophic primary infection hyphae of the biotrophic barley-powdery mildew pathogen (Fig. 3) and the hemibiotrophic fungus, *Colletotrichum destructivum*, respectively, subsequent to successful host cell entry [29,47]. In the case of the barley–powdery mildew interaction, actin filaments not only encase the haustorium but also form a ring-like structure at the tip of the emerging haustorium [29]. Such an arrangement is somewhat reminiscent of the host "prepenetration apparatus" formed during symbiotic plant–mycorrhiza interactions (see below) and may indeed fulfill a similar function by "guiding" the nascent haustorium. These findings indicate that the role of actin filaments may extend beyond mere defense functions in plant–pathogen interactions. It is conceivable that the cytoskeleton serves a dual role: while it likely initially contributes to pre-invasive defense of the attacked cell, it might be corrupted by the invader for pathogenicity functions following successful host cell invasion, e.g. for the spatial arrangement of feeding structures and/or the delivery of molecules towards the microbial feeding organs. Although this hypothesis awaits experimental verification, it is consistent with the observed enhanced transcript accumulation of an actin-encoding gene during a compatible plant–microbe interaction [70].

8. The plant cytoskeleton in rhizobial symbiosis

Bacteria from different families—including rhizobia, bradyrhizobia and others—form symbiotic interactions with plants of the legume (Fabaceae) family [71]. During this symbiosis bacteria fix atmospheric nitrogen in indeterminate nodules, derived from root cortical cells, and provide it as ammonium for use by the plant [72]. A prerequisite for establishment of this symbiotic interaction is the entrapment of bacteria between plant cell walls, within curled root hairs [71,73,74]. Subsequently, bacteria proliferate in a plant-derived infection thread (IT) that grows inside the root hair and through the epidermal cell into the cortical cell layer. Finally bacteria are endocytosed in an organelle-like membrane-coated structure, the symbiosome [75].

The phenomenon of root hair curling induced by bacterial lipochitin oligosaccharide Nod factors has been extensively studied. Spot application of purified Nod factor (NF) solution is sufficient to trigger reorientation of the root hair growth axis within a few minutes [73]. Assembly of fragmented actin filaments, observed as diffuse rhodamine–phalloidin staining, after NF treatment appears to precede the reorientation and curling of the plant root hair [76–78]. In this context, it was shown that bean root hair cells respond more rapidly in terms of actin fragmentation to methylated *Rhizobium etli* NFs than to non-methylated NFs [79]. Similarly, fragmentation and subsequent rearrangements of microtubules were observed in cortical cells of *Vicia hirsuta* and *Pisum sativum* inoculated with rhizobia or purified NFs [80,81]. *Rhizobium meliloti* Nod factors provoke disintegration of microtubules and subsequent formation of a microtubular ring structure at the root tip of chemically fixed emerging root hairs of *Medicago sativa* [82]. Nod factor application in growth-arrested root hairs resulted in a less dramatic transient microtubule assembly that laid out the angle of the new outgrowth of the root hair [83]. In an elegant study, time-lapse confocal imaging of single microtubule dynamics in young living root hairs of *Lotus japonicus* expressing a green fluorescent protein (GFP)-α-tubulin 6 fusion protein revealed an increase in short, transversally oriented and less stable microtubules as a first biological response to *Mesorhizobium loti* infection. This rearrangement preceded alteration of cell polarity and root hair curling [84]. In contrast, microtubules in non-inoculated root hair cells exhibited high growth rates and a low frequency of fragmentation, resulting in persistent microtubule growth. It appears that the plant cytoskeleton needs to be disintegrated prior to a change in cell polarity and the initial uptake of the bacteria.

Fragmentation of microfilaments and microtubules prior to root hair curling is consistent with an initial down-regulation of genes encoding cytoskeleton-modifying proteins as has been observed in transcriptional profiling of the early phase of the interaction between *Medicago truncatula* and *Sinorhizobium meliloti* [72]. Interestingly, some genes encoding components of the cytoskeleton, including β-tubulin homologs, are transcriptionally upregulated at 24 h post-inoculation, coinciding with development of the infection thread [72]. Around the IT, dense cytoskeletal elements have been identified in columns of streaming cytoplasm that connect the nucleus to the growing IT [85]; a phenomenon bearing similarity to the prepenetration apparatus that is produced during plant colonization by

endosymbiotic fungi (see below). Unlike the reported fragmentation of actin in response to bacterial NFs, increased stability of actin through monoubiquitination in root nodules of *Phaseolus vulgaris* in response to a yeast-derived elicitor has been observed [43].

9. The plant cytoskeleton in mycorrhizal symbiosis

The nitrogen-fixing symbiosis between rhizobia and legumes shares both evolutionary and developmental aspects with arbuscular mycorrhizal (AM) symbiosis [86]. AM symbiosis presumably dates back to the land colonization of plants 400 million years ago [87]. In this mutualistic endosymbiosis, fungi of the phylum *Glomeromycota* inhabit root cortical cells of most taxa of vascular plants. The fungus transfers nutrients from the soil to the root and in return is supplied with photosynthates from the plant [88]. Generally, AM fungi form hyphopodia to penetrate the root surface and grow intercellularly through the outer cortex. Upon reaching the inner cortex, the fungus forms highly ramified feeding structures, termed arbuscules, which resemble haustoria from biotrophic fungi and oomycetes [89,90].

Recently, *in vivo* imaging using GFP-labeled markers that highlight cytoskeletal structures and the endoplasmic reticulum (ER) in *M. truncatula* root clones revealed extensive nucleus-directed rearrangements of the plant cytoskeleton before and after penetration of the *Gigaspora* fungus [89]. Concomitantly with repositioning of the epidermal cell nucleus below the site of hyphal contact, microtubules form a dense subconical set and actin filaments assemble into a radial array at the contact site [89]. Subsequent movement of the nucleus to the cell cortex initiates formation of a cytoplasmic column comprised of a high-density array of microtubules and microfilaments that run parallel to the column. This so-called prepenetration apparatus (PPA) defines the transcellular pathway that the fungal hyphae follow after penetration of the cell [89] (Fig. 4). Similar dynamic nuclear movements first towards and then away from the fungus has also been reported for cowpea in the compatible pathogenic interaction with *Uromyces vignae* [28,91].

Arbuscule formation in the inner cortical cells likewise involves restructuring of the plant cytoskeleton. Using indirect immunofluorescence, different structures were observed for microfilaments and microtubules in arbuscule-containing plant cells [92,93]. While microfilaments form a dense network around arbuscule branches and the plant nucleus, short microtubules seem to bridge adjacent fungal branches and connect the arbuscule to the cell periphery [92]. Cytoskeletal rearrangements during colonization of AM fungi likely depend on increased biosynthesis of actin and tubulin as transcripts of actin, α- and β-tubulin genes were found to be upregulated in PPA-containing cells relative to non-inoculated controls [93]. In agreement with a substantial role for tubulin in PPA formation, expression levels of β-tubulin were most strongly induced.

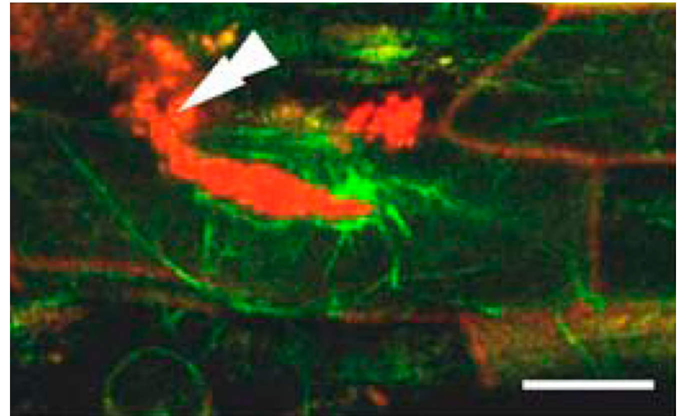

Fig. 4. Actin filaments form part of the prepenetration apparatus around an invasive hypha of *G. gigantea* in a root epidermal cell of *M. truncatula*. After penetration of the host cell the fungal hypha (indicated by red autofluorescence) remains surrounded by the constituents of the prepenetration apparatus. This structure comprises mircrotubular arrays, microfilament bundles and the ER. Exemplarily, the actin bundles (labeled with GFP:Fimbrin1-ABD, shown in green) around the hypha are depicted. The image is a *z*-axis projection of serial optical sections. Double arrowheads indicate the penetration site. Bar, 20 μm (reproduced with kind permission from [89]; copyright by the American Society of Plant Biologists).

Accumulation of actin and tubulin transcripts was less pronounced in the *M. truncatula dmi3-1* mutant cells that cannot form a PPA when compared to non-inoculated *dmi3-1* cells [93]. It would be intriguing to use real-time confocal imaging of GFP-labeled cytoskeletal elements to examine potential analogies between the structures inside the rhizobial infection thread and the mycorrhizal PPA.

Unlike in the case of plant–pathogen interactions, pharmacological inhibitors have not been comprehensively used to probe the requirement of the plant cytoskeleton in establishing symbiotic relationships. In fact, to our knowledge, only one study at present reports about such experiments. Timonen and co-workers found that disruption of host actin filaments but not microtubules reduced colonization by mycorrhizal fungi and the formation of arbuscules in tomato roots [94]. These results suggest that the actin cytoskeleton contributes to the establishment of symbiotic interactions while the microtubular network appears dispensable. Further work is clearly necessary to corroborate and extend these data in other experimental systems and by other compounds and/or methods.

10. Microbial effectors and toxins that interfere with the plant cytoskeleton

It is conceivable that part of the manifold rearrangements of microtubules and microfilaments induced by microbial invasion of plants are the consequence of secreted toxins and effector proteins of the invader, especially at the later stages of infection. A range of effectors from mammalian pathogenic bacteria are known to target the host cytoskeleton either directly by covalently modifying actin or indirectly by manipulating regulatory

proteins like small GTPases (reviewed in [95]). Often manipulation of host cytoskeleton elements is used to gain entry into the host cell. *Salmonella enterica* secretes type III effector SopE that serves as a guanine–nucleotide-exchange factor and activates the GTPase CDC42 thereby inducing the generation of actin ruffles, which eventually internalize the bacteria [96–98]. Instead of actin activation, *Shigella* spp. uses the opposite strategy, namely depletion of microtubules at the site of invading bacteria by direct interaction of its effector VirA with tubulin heterodimers to promote destabilization of the host cell at the entry site [99].

Direct experimental evidence for analogous effector activities in plant-colonizing microbes is lacking to date. Nevertheless, there are a few hints for microbial interference with the plant cytoskeleton. Firstly, several effector proteins from bacteria, including the plant–pathogen *Pseudomonas syringae* and the plant symbionts *Rhizobium* sp. and *Bradyrhizobium japonicum*, belong to the YopT family of effectors [100]. YopT is secreted by the mammalian pathogen *Yersinia pestis*. It causes cell death in yeast and rounding of HeLa cells by disrupting the actin cytoskeleton, presumably through targeting the small GTPase RhoA [100]. Both YopT and *P. syringae* AvrPph were demonstrated to function as cysteine proteases [100]. However, the substrate-specificity of AvrPph remains to be determined. Secondly, a toxin contained in the supernatant of cultured *Verticillium dahliae* (VT toxin) causes rapid fragmentation of actin filaments, an effect that was reversible after washout of the toxin [101]. The exact nature of the VT toxin, the physiological concentration secreted by the fungus and its biological relevance during plant colonization remains to be unraveled. Another phytotoxin, rhizoxin, evokes the symptoms of rice seedling blight usually caused by infection with *Rhizopus microsporus* through binding to rice β-tubulin, thereby inhibiting mitosis and causing cell cycle arrest. Surprisingly, it was recently discovered that rhizoxin is not secreted by the fungus, but from an endosymbiotic bacterium [102], thus adding a new level of complexity to this plant–microbe interaction. Lastly, the bacterial effector AvrPto has been implicated in suppressing host callose deposition, a common response upon microbial encounters that is believed to contribute to plant defense [103]. The suppression of callose deposition is possibly carried out through interaction with small Ras-related Rab GTPases [104], which are known to control vesicular trafficking (reviewed in [105]). Taking into account that the mode of action of most effector proteins that have currently been isolated from plant-invading microbes is not known, it remains a future challenge to identify those effectors directly or indirectly targeting the plant cytoskeleton.

11. The microbial cytoskeleton

Although the rearrangements in plant cell structure before and during microbial invasion have been studied in considerable detail, much less attention has been paid to the organization of the microbial cytoskeleton during infection. Polarized hyphal growth is indispensable for plant-colonizing fungi. It is associated with dynamic accumulation of vesicles, ribosomes and cytoskeletal elements in the hyphal tip, the so-called Spitzenkörper [106]. It is postulated that the molecular composition of the Spitzenkörper changes during invasive fungal growth due to vesicular trafficking [107]. Indirect immunofluorescence microscopy showed that the actin cap in hyphae of the ectomycorrhizal fungus *Amanita muscaria* was more active during increased hyphal branching induced by the helper bacterium *Streptomyces* sp. [108]. At the stage of invasive hyphal growth an F-actin-depleted zone in phalloidin-stained invasive hyphae of the oomycete *Phytophthora cinnamomi* was observed [109]. Transmission electron microscopy of these invasive hyphae led to the assumption that the actin-depleted zone is the result of vesicle accumulation in the hyphal apex [109]. A further indication of the importance of the microbial cytoskeleton is that plant defensins appear to target cytoskeletal components. An antimicrobial peptide from *Pharbitis nil*, Pn-AMP1, induces depolarization of actin, thereby leading to growth arrest in yeast [110]. However, the effectiveness of Pn-AMP1 in the plant–pathogen interaction remains to be determined.

Notably, the level of actin gene expression does not change in mycorrhizal fungi during plant colonization [108,111] while transcript levels of *A. muscaria* tubulin *AmTuba1* correlate with the amount of mycelial growth [112]. The latter is in accordance with a presumed role of microtubules in the long-distance transport of vesicles and nuclei in fungal hyphae [113], herein differing from vesicular transport in plants, which is supposedly facilitated via actin filaments [12,13]. Additionally, cytoskeletal elements might play a direct role in host penetration by pathogenic fungi. Absence of penetration peg formation in the non-pathogenic *mst12* mutant of the rice blast fungus *M. grisea* was associated with defects in tubulin reorganization, observed by confocal microscopy as Benomyl-insensitive spherical bodies of GFP-tagged β-microtubules in mature *mst12* appressoria [38]. In contrast, wildtype *M. grisea* developed a vertically oriented array of microtubules that is unique to mature appressoria. This microtubular array, in combination with actin filaments, which have also been observed in penetration pegs of the rice blast fungus by indirect immunofluorescence microscopy [114], might indicate the site of penetration peg formation. Intriguingly, *mst12* mutants were also impaired in invasive growth when inoculated through wounds [115], again underlining the importance of the cytoskeleton for invasive fungal growth.

Recently, the group of Gero Steinberg has made an effort to elucidate the molecular basis of the crucial switch from yeast-like to hyphal growth of the corn smut fungus *Ustilago maydis*—a prerequisite for mating and subsequent filamentous plant invasion of this dimorphic fungus.

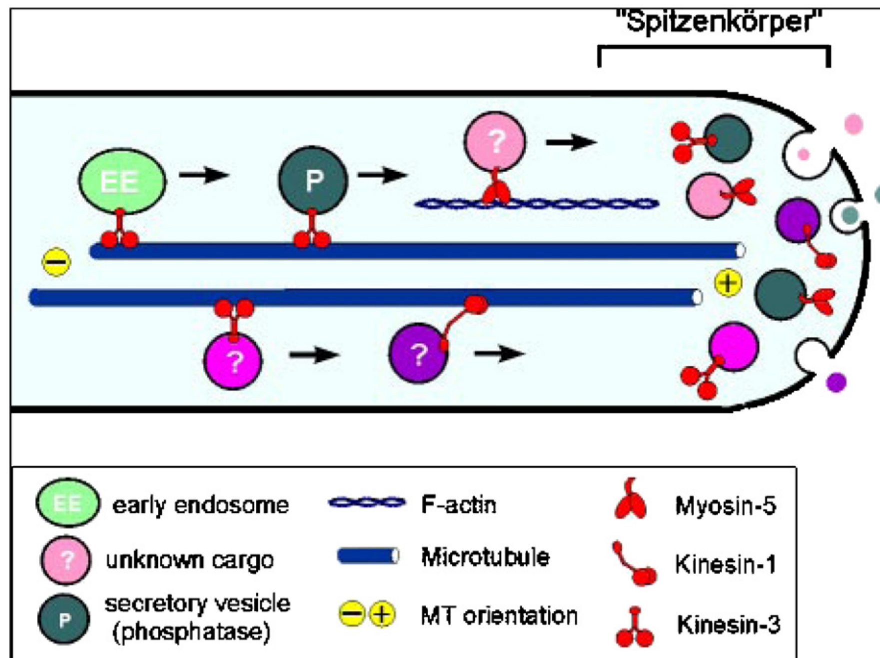

Fig. 5. Scheme of hyphal tip growth in *U. maydis* and the involvement of Kinesin-1, Kinesin-3, and Myosin-V. Long-distance transport of vesicles to the expanding hyphal tip is facilitated by the cytoskeleton binding proteins Myosin V, Kinesin-1 and Kinesin-3. Secretory vesicles and attached motors cluster in the Spitzenkörper at the hyphal apex, which presumably mediates local exocytosis guiding tip growth. Elimination of both Myosin V and either Kinesin-1 or Kinesin-3 abolish polarized growth of the fungal hypha. Kinesin-1 and Kinesin-3 likely transport vesicles and early endosomes along microtubules. The latter are oriented with their plus ends towards the growing tip. Myosin-V delivers cargo along microfilaments to the fungal Spitzenkörper. (Reproduced with kind permission from [117]).

Both microtubules and microfilaments were found to be required for maintenance of polar hyphal growth. Treatment with latrunculin A, an inhibitor of actin polymerization (Table 1), completely abolished polar growth and conjugation tube formation [113]. The molecular motor controlling the actin-dependent formation of the conjugation tube likely is Myosin-V. Immunofluorescence studies revealed that Myosin-V, actin and Kinesin1 colocalize in the Spitzenkörper of the fungal hyphae [116,117] (Fig. 5). Loss of Myosin-V did not affect tip growth in conidia and hyphae. However, mutants were swollen, not able to form conjugation tubes and seemed to be impaired in perception of the pheromone secreted by compatible strains to initiate mating [116]. The few dikaryotic hyphae formed between compatible haploid partners of Myosin-V mutants were able to penetrate host tissue but did not exhibit invasive growth, suggesting that Myosin-V is crucial for later steps of fungal pathogenesis [116]. Apart from multiple genes encoding conventional chitin synthases, filamentous fungi harbor a gene encoding a chitin synthase with an N-terminal myosin-like motor domain. These so-called class V chitin synthases are thought to move along actin filaments towards the hyphal tip, the site of particularly active chitin biosynthesis. However, direct experimental evidence for actin-dependent intracellular shuttling of these proteins is lacking to date. Fungal mutants defective in class V chitin synthases are generally apathogenic, suggesting that the actin cytoskeleton may indirectly contribute to focally localized chitin biosynthesis, which in turn appears to be required for fungal pathogenesis [118–120].

Microtubules, on the other hand, seem to be required for long-distance growth as demonstrated by treatment with the tubulin-depolymerizing drug Benomyl, which results in bipolar hyphae that remain short [113]. Two molecular motors involved in tubulin-dependent transport in *U. maydis* have been identified. Both *Kinesin1* and *Kinesin3* transcripts were found to be upregulated during hyphal growth and mutants lacking either one or the other kinesin resembled Benomyl-treated cells [117]. Ultimately, a double mutant lacking Kinesin1 and Myosin-V exhibited loss of polarity as well as impaired morphology of *U. maydis* hyphae [117]. There is also direct and indirect evidence accumulating that microtubules in growing hyphae are involved in endosomal recycling [121] and the transport of RNA [122]. Future challenges for understanding the role of microtubules include the identification of vesicle cargo shuttled along these cytoskeletal elements.

12. Outlook

In the last few years we have learned that plant cytoskeleton functions are crucial for plant defense. Likewise, the role of the cytoskeleton in microbial morphogenesis and development was discovered. In contrast, although based on descriptive reports and one pharmacological

study a key role for the plant cytoskeleton in the intracellular accommodation of symbionts appears likely, there is currently only sparse experimental support for this hypothesis [94]. Further studies employing pharmacological and/or genetic interference with the plant cytoskeleton in the context of plant–symbiont interactions are thus urgently required to fill this gap. It remains another future challenge to explore the supposed inventory of proteinaceous and non-proteinaceous effectors that pathogenic and symbiotic microorganisms employ to manipulate the plant cytoskeleton. On the other hand, it will be important to find out whether plants generally target the microbial cytoskeleton for defense. If so, what is the repertoire of defensive proteins and metabolites that exert such a function and how do they act? A further question concerns the post-invasive role of the plant cytoskeleton. Has it a function in guiding and stabilizing intracellular infection structures such as haustoria and arbuscules? Does it contribute to the delivery of nutrients to the microbe? So far no plant mutant in a gene encoding a cytoskeleton-associated protein has been described that has a defect in defense or the establishment of a symbiotic interaction. This likely reflects the essential roles of such proteins in general cellular processes and the genetic redundancy of many of these components. Novel tools that may help to overcome these limitations in the future comprise (inducible) genetic interference with cytoskeletal functions. Recently, the ethanol-inducible expression of Arabidopsis *Actin Interacting Protein 1* (*AIP1*) has been reported to disrupt actin organization in all studied cell types [123]. Likewise, overexpression of ADFs has been shown to effectively abolish cellular actin functions [53]. It would also be highly desirable to identify novel drugs that selectively interfere with the plant cytoskeleton. While specific substances such as Benomyl (Table 1) and Morlin [124] exist that selectively target fungal or plant microtubules, respectively, analogous compounds for the selective interference with microfilaments are missing to date. There is also need for further genetically encoded fluorescently labeled probes that decorate cytoskeletal elements or subpopulations thereof with minimal perturbation of cytoskeletal functions. In combination with state-of-the-art non-invasive imaging techniques, such tools would help addressing some of the questions raised above.

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