Dynamic cellular responses in plant–microbe interactions

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Encounters between plant cells and both 'friendly' and 'hostile' microbes (such as those in symbiotic and pathogenic interactions, respectively) trigger a range of highly dynamic plant cellular responses. These include reorganization of the cytoskeleton, organelle translocation, vesicle trafficking, and alterations in subcellular protein localization. Recent progress in this borderland that bridges the fields of plant–microbe interactions and cell biology heralds the transition from descriptive phenomenology to the identification and characterization of key molecules that are involved in these processes. Intriguingly, molecular events that occur in plant cells in response to microbes also take place upon abiotic wounding and during fundamental plant developmental processes, such as the tip growth of pollen, root hairs and trichomes. Thus, elementary 'activity modules' that are required for the generation of cell polarity in plant morphogenesis appear to be re-used in both abiotic and biotic stress response pathways.

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Introduction

Both aerial and subterranean plant organs are constantly exposed to intimate contacts with a plethora of microorganisms, including members of phyla as diverse as viruses, bacteria, oomycetes, fungi, and eukaryotic protozoans. The outcome of interactions between plants and microbes can be neutral, detrimental or even beneficial for the photoautotrophic organisms. Disadvantageous encounters typically manifest themselves as disease, which in extreme cases can result in full collapse of plant tissues. By contrast, benign contacts usually give rise to symbiotic relationships that typically support the plant's nitrogen metabolism and mineral uptake. Surprisingly, although many microbes have a principal phytopathogenic potential, the majority of interactions between plants and microbes remain macroscopically symptomless. This effect is at least partially based on an elaborate surveillance system that allows plants to recognize and effectively respond to most 'wanna-be' pathogens, a phenomenon frequently termed 'non-host resistance' (reviewed in [1]). Despite the fundamentally different final outcome of the three types of microbial contacts, plant cells exhibit a surprisingly similar set of highly dynamic cellular responses during these contacts. These activities ultimately lead to extensive polarization towards the microbe at the single-cell level [2]. Recent studies provide surprising insights into the molecular basis and possible biological significance of some of these highly dynamic cellular processes.

Vesicle transport and secretion

Despite extensive descriptive work in the past (e.g. [3]), host vesicle transport and exocytosis recently regained increasing attention in the field of plant–microbe interactions. This interest was initially fuelled by the pivotal finding that members of the superfamily of soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptor (SNARE) polypeptides decisively contribute to limiting host cell entry by powdery mildew fungi both in monocot and dicot plant species. SNARE proteins are known to mediate membrane fusion events in yeast and animal cells [4]. Mutations in the gene that encodes the plasma membrane (PM)-resident *Arabidopsis At*PEN1 target membrane SNARE (t-SNARE, also referred to as syntaxin) allow enhanced cell invasion by the grass powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) [5]. In *Arabidopsis*, the majority of penetration attempts by this 'inappropriate' pathogen usually fail at the cell wall. In *Atpen1* mutants, however, a significant proportion of sporelings succeed in entering the host cell, suggesting that t-SNARE function is essential to arrest fungal ingress at the cell periphery [1,5,6]. Likewise, a mutation in barley *HvRor2* partially restores susceptibility of otherwise fully resistant barley *mlo* mutants [5]. A further SNARE protein, SNAP-25, is known to form binary complexes with syntaxins to promote membrane-fusion events in animal and yeast cells [4]. Gene silencing of a barley homolog of SNAP-25, *HvSNAP34*, resulted in partially compromised *mlo* and non-host resistance, further corroborating a potential role for the secretory machinery in plant defence [5,7]. Notably, the expression of the corresponding *Arabidopsis* homolog, *AtSNAP33*, is highly responsive to various pathogens and mechanical stimulation [8]. The microscopically visible and diaminobenzidine-positive (indicating H_2O_2accumulation) vesicle-like bodies

(VLBs) frequently seen around attempted host cell entry sites in the interaction between barley and *Bgh* (e.g. [5]) were recently analyzed at the ultrastructural level. These studies revealed that VLBs actually encompass a variety of structures that are distinct from individual vesicles, including small cell wall appositions (papillae), paramural bodies, multivesicular bodies (MVBs) and osmophilic bodies (Q An, R Hückelhoven, K-H Kogel, AJE van Bel, pers. comm.).

A potential involvement of PM-resident syntaxin(s) in plant immunity is further supported by the observation that the *At*SYP122 t-SNARE is rapidly phosphorylated upon treatment of *Arabidopsis* cultured cells with Flag22 (flg22) [9], a highly conserved 22-mer peptide derived from the bacterial flagellum that acts as general elicitor of a set of defence-related responses in plants [10]. Likewise, rapid and transient phosphorylation of a syntaxin occurs in the context of the race-specific *Avr9–Cf-9* interaction in tobacco [11]. Syntaxin phosphorylation has been detected immunologically by a shift in protein mobility in western blot analysis and shown to require presence of both the *Cf-9* resistance gene and the *Avr9* avirulence determinant. Notably, syntaxin phosphorylation occurred only upon treatment with the race-specific *Avr9* elicitor and not upon application of the general elicitor flg22 [11]. The biological relevance of the phosphorylation of the syntaxins *At*SYP122 and *Nt*SYP in the course of plant–microbe interactions remains to be shown.

Recently, a further *Arabidopsis* PM-resident syntaxin, *At*SYP132, was found to be phosphorylated in response to flg22 (M Kalde, TS Nühse, S Peck, pers. comm.). Lack of T-DNA knockout mutants in *AtSYP132* prompted Kalde *et al.* to employ virus-induced gene silencing (VIGS) of the respective tobacco ortholog, *NbSYP132*, in *Nicotiana benthamiana* to study the biological role of this syntaxin during plant–microbe interactions. VIGS identified this gene as a component that is essential for restricting bacterial growth and suppressing disease symptoms during AvrPto–Pto-mediated isolate-specific resistance to *Pseudomonas syringae* pv. *tabaci*. Proteomic and immunological analysis of apoplastic fluids revealed that secretion of at least two pathogenesis-related (PR) proteins is compromised in *NbSYP132*-silenced plants, suggesting that *Nb*SYP132 is required for the exocytosis of a subset of PR proteins.

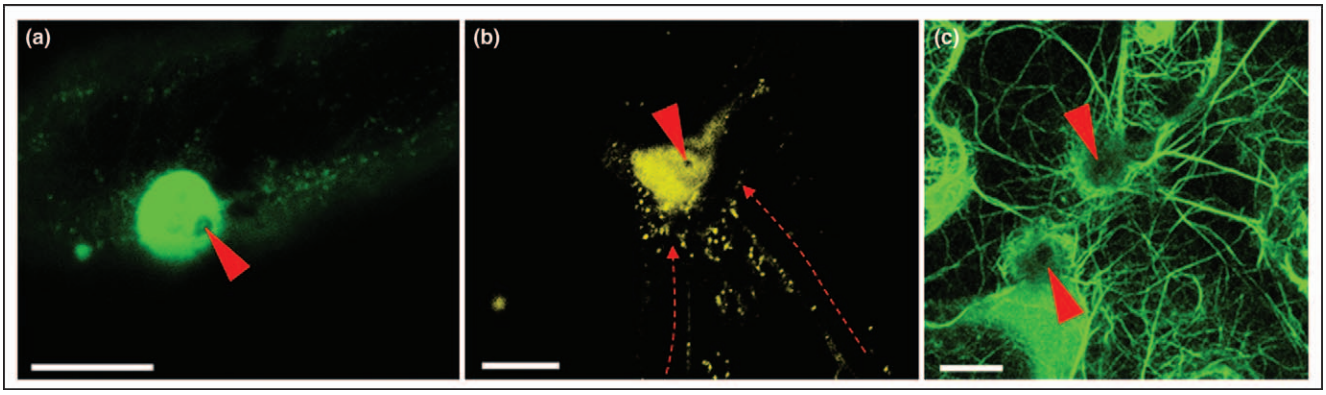
Indirect evidence for a role of exocytosis in non-host resistance is also provided by a study on plant–bacteria interactions. Secretion of a cocktail of metabolites that have antimicrobial activity confers tissue-specific resistance to a range of bacterial microbes in the roots of *Arabidopsis*. Interestingly, a *P. syringae* strain that is partially resistant to these compounds and also able to block their synthesis and/or secretion is able to colonize root tissues and to cause disease. The capability to overcome this exudation-based mechanism depends on an intact bacterial type III secretion system, suggesting that this strain has evolved a mechanism that uses secreted microbial effectors to suppress root exudation [12•].

Activation of the secretory pathway also appears to represent a fundamental step of systemic acquired resistance (SAR), a plant-wide immune response that is triggered upon a local stimulation of the plant's pathogen surveillance system. NON-EXPRESSOR OF *PR* GENES 1 (NPR1), a major control element of SAR in *Arabidopsis*, is a transcription regulator that resides in the cytoplasm of unchallenged cells but enters the nucleus upon activation of the SAR pathway. An elegant gene expression survey revealed various *PR* genes and a set of genes that encode key elements of the secretory pathway (including a range of chaperones and co-chaperones) as immediate downstream targets of NPR1 [13••]. The findings indicate an enhanced cellular requirement for the secretory machinery and its protein-folding mediators in the course of SAR, which is probably due to an enhanced exocytotic flux of PR proteins.

Reorganization of the plant cytoskeleton and organelle positioning

Numerous descriptive and/or pharmacological inhibitor studies previously demonstrated the importance of cytoskeletal re-arrangements for the execution of pathogen entry control at the cell periphery [2,14•]. Although changes in the organization of the plant cytoskeleton during plant–microbe interactions are complex and varied, actin microfilaments in particular appear to play a pivotal role in the timely and spatial recruitment of the plant's defensive forces at infection sites ([14•]; Figure 1). Recent work employing a series of green fluorescent protein (GFP)-tagged organelle reporter lines allowed *in vivo* monitoring of cell dynamics in response to inoculation with compatible and incompatible oomycete pathogens [15]. Strikingly, in all genotype combinations, actin microfilament organization was resolved and focally reorganized towards sites of attempted penetration, whereas microtubule organization appeared to be affected only subtly. Interestingly, the observed radial microfilament reorganization coincided with stable ER aggregations and saltatory Golgi body accumulation at pathogen penetration sites. The latter stop-and-go movement might indicate site-directed secretion events, and it is conceivable that a pathogen-responsive actin filament network could indeed provide the routes required for the observed organelle positioning, vesicle trafficking and syntaxin-mediated exocytosis postulated above. Consistent with this idea, interference with the responsiveness of the microfilament network by application of actin polymerization inhibitors (e.g. cytochalasin) resulted in enhanced pathogen entry in various compatible and incompatible plant–pathogen combinations [2,16,17]. Notably, cytochalasin treatment even compromises the control of

Figure 1



Pathogen-triggered cell polarity. Confocal imaging of fluorescent marker protein fusions in transgenic *Arabidopsis* plants reveals pathogen-induced dynamic changes in subcellular protein localization, vesicle transport processes and the reorganization of cytoskeleton architecture. **(a)** Focal accumulation of GFP-labelled syntaxin PEN1 in a lipid raft-like plasma-membrane microdomain triggered by attempted penetration (red arrowhead) of the inappropriate pea pathogen *Erysiphe pisi*. **(b)** Vesicle-like structures tagged with yellow fluorescent protein (YFP)-labelled *Arabidopsis* R-SNARE VAMP722 move to (dotted red arrows) and accumulate at sites of interaction (red arrowheads) with the pea powdery mildew *Erysiphe pisi*, suggesting polarized secretion processes. **(c)** GFP-tagged actin microfilaments (decorated by a mouse Talin–GFP fusion protein) focusing on attempted penetration sites (red arrowheads) of the non-adapted pathogen *Colletotrichum truncatum*. This image was kindly provided by Yoshitaka Takano, Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan. Please note that fungal infection structures are not visible in these confocal micrographs. The scale bar in all images represents 20 μm.

pathogen entry into dedifferentiated tobacco BY-2 cells that are challenged by compatible and inappropriate powdery mildew species [18].

The importance of these actin-related mechanisms for host-range demarcation against inappropriate pathogens was impressively demonstrated in *Arabidopsis* plants harbouring knockout mutations in the gene that encodes the lipase-like protein ENHANCED DISEASE SUSCEPTIBILTY 1 (EDS1). Mutations in *EDS1* alone only partially compromise the non-host resistance of *Arabidopsis* against the wheat powdery mildew fungus, *Blumeria graminis* f. sp. *tritici* (*Bgt*). Additional pharmacological inhibition of actin polymerisation in *eds1* mutant lines, however, fully wipes out non-host resistance and allows completion of the pathogen's infection cycle [16].

Although these experiments corroborate the importance of adaptive cytoskeleton remodelling in efficiently defying potential pathogens, the molecules that signal and bring about the dramatic reorganizations are still elusive. Evidence for a possible role for RAC/ROP family G-proteins in pathogen-induced actin architecture modifications comes from transient gene-expression analyses in barley. Plant RAC/ROP G-proteins were recently shown to be required for efficient colonization of barley plants by *Bgh*, suggesting that these polypeptides function as potential host susceptibility factors [19]. Moreover, overexpression of a constitutive active variant of the barley RAC/ROP G-protein RACB (caRACB) partially inhibited actin remodelling and enhanced fungal penetration success [20•]. By contrast, transient gene silencing of *RacB* resulted in marked actin polarization, coincident with significantly enhanced penetration resistance [20•]. Taken together, these results suggest that RAC/ROP G-proteins are potent regulators of pathogen-induced plant microfilament reassembly that might be targeted by fungal pathogens to establish compatibility. In addition, Opalski and co-workers [20•] provide evidence that another plant susceptibility factor, the barley MLO protein, also affects actin-dependent control of pathogen entry at the cell periphery. The presence of MLO in susceptible barley wildtype plants correlates with a reduced and delayed focusing of actin in *Bgh*-challenge experiments.

These findings were supported and expanded by a recent series of experiments that utilized pharmacological and genetic approaches. Upon cytochalasin E treatment, the entry success of barley powdery mildew was significantly enhanced in both wildtype barley plants (which had 'super-accessibility') and otherwise resistant *mlo* mutants, reinforcing the notion that cytoskeleton function is necessary for basal and *mlo*-dependent broad-spectrum disease resistance. Interestingly, these results could be phenocopied by ectopic expression of genes that encode the plant actin depolymerising factor (ADF). Reminiscent of pharmacological inhibitor effects, the perturbation of the actin cytoskeleton via ADF overexpression also severely affects barley non-host penetration resistance to the inappropriate wheat and pea powdery mildews *Bgt* and *Erysiphe pisi*, respectively (M Miklis, V Lipka, RA Bhat, P Schulze-Lefert, R Panstruga, unpublished). Intriguingly, Miklis and co-workers have evidence that

genetic interference with the actin cytoskeleton, although initially enabling enhanced host-cell entry, negatively impacts the mid- to long-term maintenance of normally compatible biotrophic interactions. In compatible interactions, developing fungal feeding organs (haustoria) are indeed associated with confined circular actin filament structures and are targets for individual filament cables [20•]. This suggests that haustorium formation and/or nutrient supply might rely on cytoskeleton-dependent processes. Thus, the actin cytoskeleton could play a dual, Janus-faced role in compatible interactions between host plants and biotrophic fungal pathogens: first a defence role that subsequently revolves to assist pathogenesis.

Additional evidence for variable modifications of cytoskeleton architecture in the course of pathogen infection has been provided by experiments monitoring talin–GFP-labelled actin in tobacco host and non-host interactions with the hemibiotrophic ascomycetes *Colletotrichum destructivum* and *Colletotrichum graminicola*, respectively [21]. Initially, plant cells that are involved in host and non-host interactions appear indistinguishable and show the typical radial actin filament arrays along which the nucleus shuttles to sites of attempted fungal penetration. Later, the non-host interaction is characterized by efficient penetration resistance whereas the compatible combination allows host-cell entry and the formation of a biotrophic infection vesicle. During disease development, susceptible plants exhibit stage-dependent changes in cytoskeleton organization, with a concomitant decline in the integrity of the host cells' actin cytoskeleton upon the switch from biotrophy to necrotrophy.

It is conceivable that plant pathogens, in analogy to bacterial pathogens of mammals (reviewed in [22,23]), have evolved means to establish a compatible host–parasite interaction by perturbation of processes that are dependent on the actin cytoskeleton. Intracellular bacterial human pathogens, for example, inject effector proteins that impede cytoskeleton function into the host cytoplasm via the so-called type III secretion system. Plant pathogenic bacteria, although generally being non-invasive extracellular pathogens, share this apparatus for the efficient transfer of effector molecules. Interestingly, it appears that delivery of the effector AvrPto is required for the suppression of apoplastic callose deposition in leaves of susceptible *Arabidopsis* challenged with the bacterial pathogen *P. syringae* [24]. Focal callose deposition at attempted pathogen entry sites has been shown to be actin-dependent (e.g. [18]), and so it is tempting to speculate that AvrPto might impinge either directly or indirectly on the function of the host actin cytoskeleton.

Not surprisingly, the accommodation of symbiotic organisms by plants is also generally accompanied by dramatic cytoskeletal rearrangements. The well-known legume–*Rhizobium* symbiosis, for example, requires cytoskeleton modifications in the root hair and cortex cells of the host plant for root hair curling, infection thread growth and root nodule development [14•]. The changes in microfilament and microtubule organization that are necessary for early symbiotic steps such as root hair curling are known to be stimulated by rhizobial secretion of lipochitin-oligosaccharides, the host-specific nodulation (Nod) factors. Exogenous application of Nod factors to root hairs results in the rapid fragmentation of actin bundles and is accompanied by increased apical influxes and intracellular levels of calcium [25]. Chemical substituents on the Nod factors are important for their biological activity, either for host specificity or efficacy [26–28]. Recent work demonstrated that methylated *Rhizobium etli* Nod factors are more active than their non-methylated counterparts in inducing hair curling and actin cytoskeleton rearrangements in *Phaseolus vulgaris* roots [29].

Similarly, the establishment of symbiotic relationships with mycorrhizal fungi also depends on cytological modifications of the host plant's root cells. In all categories of mycorrhiza, these modifications include alterations in the organization of the cytoskeleton, which have been characterized extensively at the descriptive level [14•]. Recent work by Ditengou and colleagues [30] suggests that the ectomycorrhizal fungus *Pisolithus tinctorius* controls the rate of root hair elongation in the host plant *Eucalyptus globules* ssp. *bicostata* by delivering the indole alkaloid hypaphorine. In these experiments, application of hypaphorine dramatically changed the cytoskeletal organization of elongating host root hairs, disrupting the typical actin cap at the tip and leading to the formation of aberrant microtubules in the subapical region. These alterations are suggested to hinder the tip-directed delivery of vesicles that is necessary for elongation and could explain absence of host root hairs from mature ectomycorrhizae.

In summary, the experiments described above demonstrate that the host cytoskeleton plays a key regulatory role in biotic interactions with both pathogenic and beneficial microorganisms. As a consequence, diverse strategies to interfere with the plant's cytoskeleton by delivery of specific proteinaceous or metabolic effector molecules appear to have evolved independently in the different microbial taxa.

It is not surprising that the formation of infection structures and the site-directed delivery of effectors also involve polarization processes in the interacting microorganism. In this context, it is important to notice that the apathogenic *mst12* mutant of the rice blast fungus *Magnaporthe grisea* appears to be defective in actin and microtubule filament reorganization that is usually associated with penetration peg formation in mature appressoria [31]. Therefore, cytoskeleton-dependent processes

appear to draw up the combat lines that determine victory, defeat or mutually beneficial co-existence in plant–microbe interactions.

Dynamic changes in subcellular protein localization

The microbe-triggered global reorganization of plant cells described above is well known, but the identification of individual proteins that exhibit an altered subcellular localization upon a biotic stimulus is a fresh finding. Although it is conceivable that numerous proteins are re-localized during plant–microbe contacts, only few examples have been described to date.

The application of a peptide elicitor, Pep13, that is derived from the oomycete pathogen *Phythophtora* triggers a prototypical innate immune response in cultured parsley cells that involves the activation of three mitogen-activated protein kinases (MAPKs) by an upstream MAPK kinase, *Pc*MPKK5. Intriguingly, as shown by *in planta* immunolabelling, all three MAPKs exhibit an increase in nuclear localization upon activation, possibly facilitating their ability to mediate the phosphorylation of nuclear-localised downstream substrates [32].

The unusual *Arabidopsis* resistance protein RRS1-R confers broad-spectrum resistance to the bacterial pathogen *Ralstonia solanacearum*. RRS1-R appears to be the result of a gene-fusion event. It possesses an amino-terminal TIR–NBS–LRR (Toll-interleukin repeat–nucleotide-binding sequence–leucine-rich repeat) domain that is characteristic of many plant resistance proteins and a carboxy-terminal WRKY domain, a feature of a range of plant transcription regulators [33]. In yeast two-hybrid systems, RRS-1R interacts with its cognate avirulence protein, PopP2, an effector that is delivered by the bacterial type III secretion system. Transient co-expression of fluorescently labelled PopP2 and RRS-1R revealed that the two fusion proteins co-localized in the nucleus of *Arabidopsis* protoplasts, whereas co-expression of fluorescently tagged RRS-1R and a PopP2 variant that lacks a potential nuclear localization signal resulted in the presence of both fusion proteins in the cytoplasm [34]. It is possible that PopP2 and RRS-1R initially interact (either directly or indirectly via a third protein) in the cytoplasm and subsequently co-translocate into the nucleus.

Translational fusions of the *Arabidopsis* *At*PEN1 and barley *Hv*ROR2 syntaxins with GFP distribute evenly at the cell periphery of healthy, unchallenged plant cells. Interestingly, however, both proteins focally accumulate at prospective fungal entry sites upon inoculation with powdery mildew spores ([35•,36••]; Figure 1). Reminiscent of 'lipid rafts' in animal cells [37], the circular site of focal syntaxin accumulation defines a novel pathogen-triggered PM microdomain that harbours a subset of PM-resident proteins and excludes others [36••]. It remains to be shown whether the PM microdomain in plants is composed of a particular lipid makeup that is analogous to the proposed composition of lipid rafts in animal cells.

The PM-associated receptor-like kinase FLAGELLIN SENSITIVE 2 (FLS2) serves as an extracellular sensor in plant innate immunity. FLS2 recognizes presence of the general peptide elicitor flg22 and triggers a signal transduction cascade that results in the transcriptional activation of a range of genes [10]. Intriguingly, upon extracellular application of flg22, GFP-tagged FLS2 rapidly (within minutes) disappears from the PM and becomes visible in mobile cytoplasmic vesicle-like bodies (S Robatzek, T Boller, pers. comm.). This phenomenon is reminiscent of receptor internalization upon ligand binding in animal cells [38]. The biological role of flg22-triggered FLS2 internalization is not clear yet but might be related to receptor desensitization, receptor recycling, or the actual initiation of the signal transduction cascade in signalosome-like vesicular bodies (S Robatzek, T Boller, pers. comm.; [39]).

Nodulation Signalling Pathway 2 (*NSP2*) from *Medicago truncatula* encodes a GRAS-like transcriptional regulator that is essential for Nod-factor signalling in the symbiotic interaction between the legume and rhizobia. The ectopically expressed GFP–NSP2 fusion protein localizes to the nuclear envelope and to the endoplasmic reticulum of resting cells but relocalizes to the nuclear lumen upon Nod-factor stimulation [40••]. It remains to be shown, however, whether this is an authentic protein relocalisation or whether GFP–NSP2 in the nuclear envelope becomes degraded and replaced by *de novo*-synthesized fusion protein.

Conclusions

Dynamic cellular processes including actin cytoskeleton remodelling, organelle translocation, focal exocytosis and specific protein re-localization occur in both symbiotic and hostile plant–microbe encounters, and usually result in extensive polarization at the single-cell level (Figure 1). Similar re-shuffling activities and associated cell polarization are well-known from tip growth during plant development, occurring for example during pollen tube growth, and during root hair and trichome expansion [41]. It is thus conceivable that plant cells re-use similar 'activity modules' to generate cell polarity during developmental processes and in abiotic and biotic stress response pathways. This provocative hypothesis is supported by various observations. First, a subset of similar (although not identical) molecular and morphological alterations occur in plant cells upon local elicitor application or local mechanical stimulation, suggesting at least partial mechanistic overlap between biotic and abiotic stress response pathways [42]. Second, the *Medicago truncatula* gene *Does not Make Infections 2* (*DMI2*) encodes a receptor-like kinase (NORK) that is essential for the

establishment of a symbiotic interaction between legumes and soil-living rhizobia [43]. NORK-defective mutants of three legume species not only fail to support symbiotic relationships but also exhibit a characteristic root hair tip-growth phenotype, indicating that a common signalling pathway for proper tip growth is shared during root hair development and for entrapping symbiotic bacteria [43]. Finally, meta-analysis of publicly available *Arabidopsis* microarray data reveals that components that are genetically implicated as having antifungal properties at the cell periphery are co-expressed in various conditions (V Lipka, R Panstruga, M Humphry, M Lim, H Wei, M Stein, P Schulze-Lefert, S Somerville, unpublished). These genes encode not only MLO (*At*MLO2) and the SNARE proteins *At*SYP121 and *At*SNAP33 but also the *At*PEN2 glycosyl hydrolase and *At*PEN3 ATP-binding cassette (ABC) transporter, both of which play a crucial role in non-host defence (V Lipka, M Stein, P Schulze-Lefert, S Somerville, unpublished). Surprisingly, particularly prominent co-expression of these genes was detected when microarray data from various developmental stages/tissue types were compared using the *Arabidopsis* tissue-specific expression database (http://www.atted.bio.titech.ac.jp/). This finding suggests that this set of genes has a potential role beyond antifungal defence in plant developmental processes. It is conceivable that subtle modifications of the proposed 'activity modules' bring about the specificity that is required for each individual biological process. Possibly, a combination of specific stimuli is required to trigger a given pathway or to generate selectivity in the cargo loaded into the secretory machinery. In biotic interactions, microbial effectors might additionally modulate these cellular response units.

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