Tansley review

Protein actors sustaining arbuscular mycorrhizal symbiosis: underground artists break the silence

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Received: *11 January 2013*
Accepted: *14 March 2013*

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New Phytologist (2013) **199:** 26–40
doi: 10.1111/nph.12287

Key words: arbuscular mycorrhiza, biotrophy, carbon (C), membranes, phosphorus (P), silencing.

Summary

The roots of most land plants can enter a relationship with soil-borne fungi belonging to the phylum Glomeromycota. This symbiosis with arbuscular mycorrhizal (AM) fungi belongs to the so-called biotrophic interactions, involving the intracellular accommodation of a microorganism by a living plant cell without causing the death of the host. Although profiling technologies have generated an increasing depository of plant and fungal proteins eligible for sustaining AM accommodation and functioning, a bottleneck exists for their functional analysis as these experiments are difficult to carry out with mycorrhiza. Nonetheless, the expansion of gene-to-phenotype reverse genetic tools, including RNA interference and transposon silencing, have recently succeeded in elucidating some of the plant-related protein candidates. Likewise, despite the ongoing absence of transformation tools for AM fungi, host-induced gene silencing has allowed knockdown of fungal gene expression *in planta* for the first time, thus unlocking a technological limitation in deciphering the functional pertinence of glomeromycotan proteins during mycorrhizal establishment. This review is thus intended to draw a picture of our current knowledge about the plant and fungal protein actors that have been demonstrated to be functionally implicated in sustaining AM symbiosis mostly on the basis of silencing approaches.

I. Casting for a scenario

The intracellular accommodation of a microorganism by a living plant cell, which is referred to as a biotrophic association, can either lead to plant beneficial or detrimental effects, thus making these interactions of major importance in agriculture (Paszkowski, 2006; Gianinazzi *et al.*, 2010). Typically, most parasitic biotrophs responsible for devastating plant pathologies, such as mildews, rusts and smuts, derive nutrients from shoot tissues without having any alternative energy source. By contrast, most beneficial biotrophic microorganisms, such as mycorrhizal fungi and nitrogen-fixing rhizobia, colonize root tissues and have access to nutrients outside the plant, raising the possibility for bi-directional nutrient movement and the development of a mutualist rather than a parasitic interaction (Smith & Smith, 1990). In arbuscular mycorrhiza (AM), the symbiosis that most terrestrial plant roots engage in with soil-borne fungi of the phylum Glomeromycota, AM fungal extra-radical hyphae absorb mineral nutrients, mainly

phosphorus (P) and nitrogen (N), from the soil which are supplied to the host plant in exchange for sugars generated by photosynthesis (Parniske, 2008). Although leading to different outcomes, mutualistic and parasitic biotrophs share the ability to penetrate the plant cell through the differentiation of specialized intracellular structures corresponding to haustoria in most pathogenic interactions; haustoria are termed arbuscules in the AM symbiosis, or bacteroids in the rhizobial nitrogen-fixing (RNF) symbiosis (Parniske, 2000). Because these structures are always surrounded by a host-derived plant plasma membrane, microbial biotrophs remain separated from the host cytoplasm. A resulting common feature of plant–biotroph associations corresponds to the existence of an interface, thought to be the main site of nutrient and signal flow between cells, which comprises the plant and the microbial membranes separated by a plant-derived apoplast (Parniske, 2000; Perfect & Green, 2001). For a successful interaction, biotrophs have to either avoid or suppress plant defence reactions together with redirecting the host metabolic flow to their benefit without killing the host. The mechanisms by which this is achieved remain largely unknown but proteins happen to take the lion's share in the paradigms that currently govern plant-biotroph interactions by playing key roles in mediating recognition, signalling and nutrient transport (Schmidt & Panstruga, 2011). Although a vast majority of the crop plants used to feed the human population have AM symbiosis, the elucidation of key protein actors governing this mutualistic interaction is far from being as developed as that involved in other biotrophic systems, mainly owing to typical developmental and genetic traits.

Unlike several pathogens with a complete (*Cladosporium fulvum, Ustilago maydis*) or hemi-biotrophic (*Magnaporthe, Colletotrichum* spp.) lifestyle, AM fungi so far have proven difficult to culture in the absence of a host root. A more drastic feature limiting functional analyses in AM symbiosis relates to the fact that Glomeromycota, for which no sexual cycle is known, are unique in so far as their spores and coenocytic hyphae contain multiple nuclei in a common cytoplasm, making classical genetic approaches unsuitable and transformation attempts so far unsuccessful in generating permanent transgenic expression (Bonfante & Genre, 2010). Finally, the genomes of AMF are not assembled yet, and comprehensive predictions of putative actors sustaining symbiosis cannot be performed (Martin *et al.*, 2008). As a result, AM fungi have not entered the post-genomics era that research on many biotrophic pathogens has now achieved (Schmidt & Panstruga, 2011). Nevertheless, the application to AM symbionts of high throughput technologies including proteomics and transcriptomics, has revealed candidate symbiosis-related traits in the model AM fungus *Rhizophagus irregularis* (formerly *Glomus intraradices*) (Recorbet *et al.*, 2009; Tisserant *et al.*, 2012). The AM symbiotic programme displayed by mycotrophic host plant roots toward AM fungi is depicted as a chronological series of events including the pre-symbiotic phase, contact, fungal entrance and intra-radical fungal proliferation (Fig. 1). However, establishment of AM symbiosis in the whole root is asynchronous as all fungi developmental stages are present simultaneously. This makes difficult a specific targeting of symbiosis-related structures and processes after plant penetration (Paszkowski, 2006). Consequently, although plant mutants are key tools for the genetic dissection of mycorrhizal development, the most frequently described phenotypes so far consist of hosts compromised for root penetration events and fungal growth arrest at the plant–fungus contact stage (Marsh & Schultze, 2001; Parniske, 2008). The use of model legumes has paved the way for isolating corresponding genes from loss-of-function mutant backgrounds and defining the common SYM pathway that mediates signalling processes essential for both RNF and AM symbioses (Parniske, 2008). By reason of the evolutionary divergent nature of AM and RNF interactions, it has nonetheless been anticipated that additional and AM-specific genes might govern endomycorrhizal formation and functioning, pointing to the necessity of performing holistic approaches. In this respect, transcriptomics, proteomics and metabolomics have succeeded in shedding light on plant genes/proteins and metabolic pathway candidates for the AM symbiotic programme by virtue of their differential expression between mycorrhized and nonmycorrhized hosts. Among the numerous studies that have been performed, the most meaningful cover transcriptome changes (Liu *et al.*, 2003; Manthey *et al.*, 2004; Guïmil *et al.*, 2005; Hohnjec *et al.*, 2005; Krajinski & Frenzel, 2007), protein abundance modifications (Bestel-Corre *et al.*, 2002; Valot *et al.*, 2005, 2006; Amiour *et al.*, 2006; Aloui *et al.*, 2009; Campos-Soriano *et al.*, 2012) and metabolic reorientations (Lohse *et al.*, 2005; Schliemann *et al.*, 2006; Walter *et al.*, 2007, 2010). Additionally, the development of laser-assisted microdissection has made possible tissue-specific analyses, including transcript and proteome profiling on isolated arbuscule-containing cells (Balestrini *et al.*, 2007; Gomez & Harrison, 2009; Gaude *et al.*, 2012). As a result, an increasing depository of plant and fungal candidates eligible for sustaining the AM symbiotic programme has emerged, but a bottleneck exists for their functional analysis as these experiments are time-consuming and difficult to carry out with AM fungi (van de Wouw & Howlett, 2011). Despite these limitations, recent years have seen fascinating contributions in demonstrating the role of proteins in AM symbiosis, which have been driven by the progress made in reverse genetics and the possibility to knockdown/knockout gene function mainly by using RNA interference (RNAi) and/or transposon silencing (Bhadauria *et al.*, 2009; Nunes & Dean, 2012). This review is thus intended to draw a picture of our current knowledge about the plant and fungal protein actors that have been demonstrated to be functionally implicated in sustaining AM symbiosis, mostly on the basis of silencing approaches.

II. Nominees for a preliminary role

1. Best costume design: 'A formal dress-code: branching out for contact'

The AM symbiosis is the result of a complex exchange of molecular information that starts before the partners engage in physical contact (Bonfante & Requena, 2011). A major breakthrough in elucidating this pre-symbiotic crosstalk relates to the identification of plant root-exuded metabolites, the so-called strigolactones (SLs), which stimulate branching of germinated AM hyphae to encourage host-root colonization (Akiyama *et al.*, 2005). SLs were originally

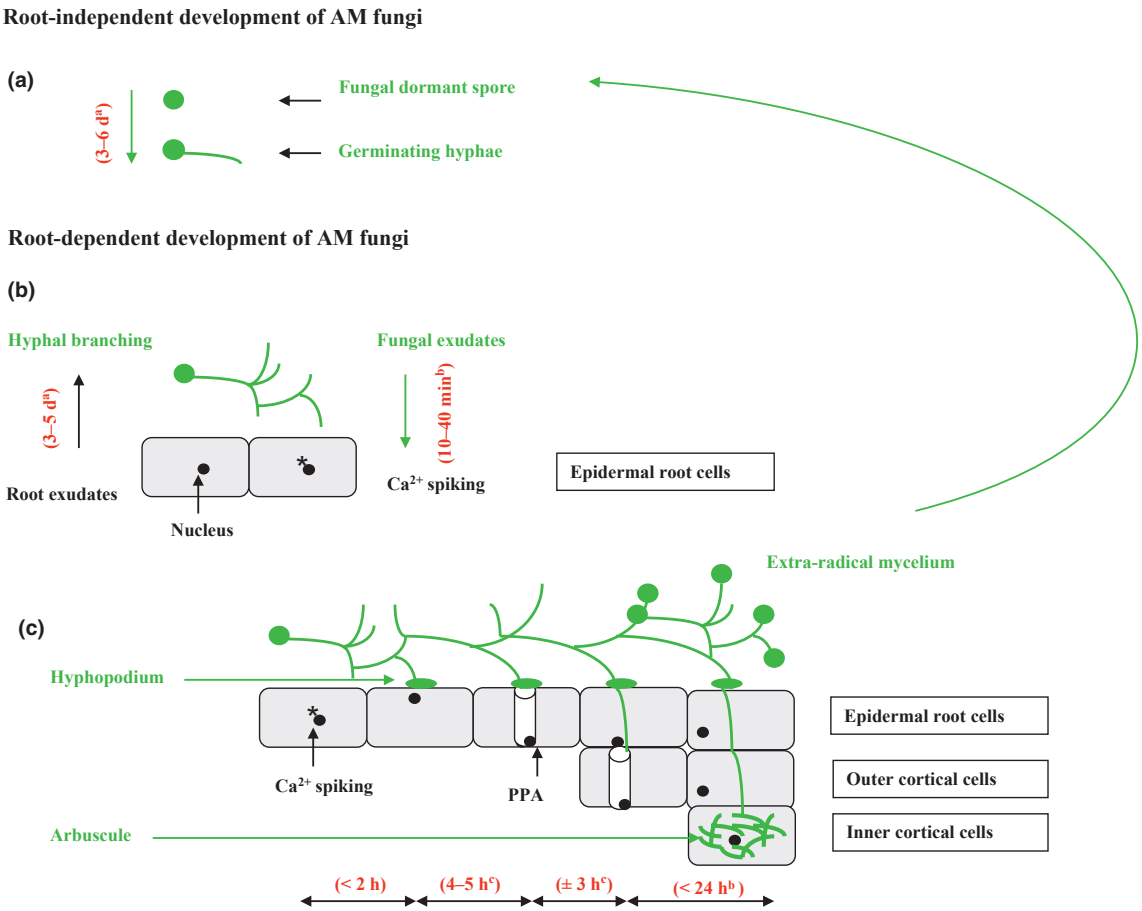

Fig. 1 Schematic overview of the life cycle of an arbuscular mycorrhizal (AM) fungus. (a) Hyphae germination at the expense of spore storage lipids. (b) In the vicinity of a host plant, root exudates trigger hyphal branching and concomitantly, the fungal exudates perceived by the host lead to calcium spiking through the activation of the common SYM pathway, which generates a reprogramming of the elicited and adjacent root cells in order to accommodate the fungus. (c) Following contact between the two partners, the AM symbiont differentiates a root-adhering hyphal structure, the hyphopodium that triggers the formation of the prepenetration apparatus (PPA) in the contacted and underlying outer cortical cells. The PPA guides the growing hypha from epidermal to inner cortical cells where a highly branched fungal structure, the arbuscule, can differentiate to form an extensive surface area for nutrient exchange. Concomitantly, the AM fungus develops an extra-radical mycelium to explore the soil for resources and new hosts. Green and black colours refer to fungal and plant structures or metabolites, respectively. The relative time-course of these events is indicated in parentheses according to: [a] Chabaud *et al.* (2002); [b] Chabaud *et al.* (2011) and [c] Genre *et al.* (2005).

identified as stimulators of the germination of root-parasitic weeds, which are obligate plant biotrophs that threaten resource-limited agriculture (Cook *et al.*, 1966). During the last decade, genes encoding enzymes essential for SL biosynthesis or perception/signalling have been identified through their cloning from a series of mutants displaying increased shoot branching phenotypes, as reviewed in Xie & Yoneyama (2010). Results showed that SLs are synthesized from a carotenoid substrate by sequential cleavages involving two carotenoid cleavage dioxygenases (CCD)7 and 8 and a subsequent oxidation by a cytochrome P450. As illustrated in Fig. 2, three other genes termed *D27*, *D14* and *D3* were identified in rice, which encode a plastid-located iron-containing protein involved in SL biosynthesis, a α/β-fold hydrolase and a F-box protein involved in signal perception, respectively (Xie & Yoneyama, 2010).

Initial functional demonstrations for a role of SLs in sustaining the AM symbiotic programme come from SL biosynthesis knockout mutants *(ccd8)* in tomato and pea, which display a reduction in mycorrhizal colonization of roots (Gomez-Roldan *et al.*, 2008; Koltai *et al.*, 2010). Likewise, significantly decreased concentrations of SL and reduced arbuscule abundance were recorded in tomato plants expressing a *cdd7* antisense construct (Vogel *et al.*, 2010). Recently, new insights into the functional characterization of the SL-dependent symbiotic signalling were obtained from silencing-based experiments performed in barrel medic, rice and petunia. In the search for efflux carriers for strigolactones, Kretzschmar *et al.* (2012) isolated ABC transporters of *Petunia hybrida* on the basis of their abundance in phosphate-starved or mycorrhizal roots. Using silencing and/or transposon-mediated loss-of-function, the candidate PDR1 was demonstrated to act as a strigolactone export carrier because only extra-radical SL concentrations (orobanchol) were affected in *pdr1* mutants, in which root exudates showed a reduced activity for stimulating hyphal branching of AM fungi relative to wild-type. Likewise, petunia *pdr1* knockout or knockdown lines displayed a significantly reduced ability to accommodate mycosymbionts without exhibiting defects in arbuscule morphology, suggesting that quantitative differences in colonization were due to decreased

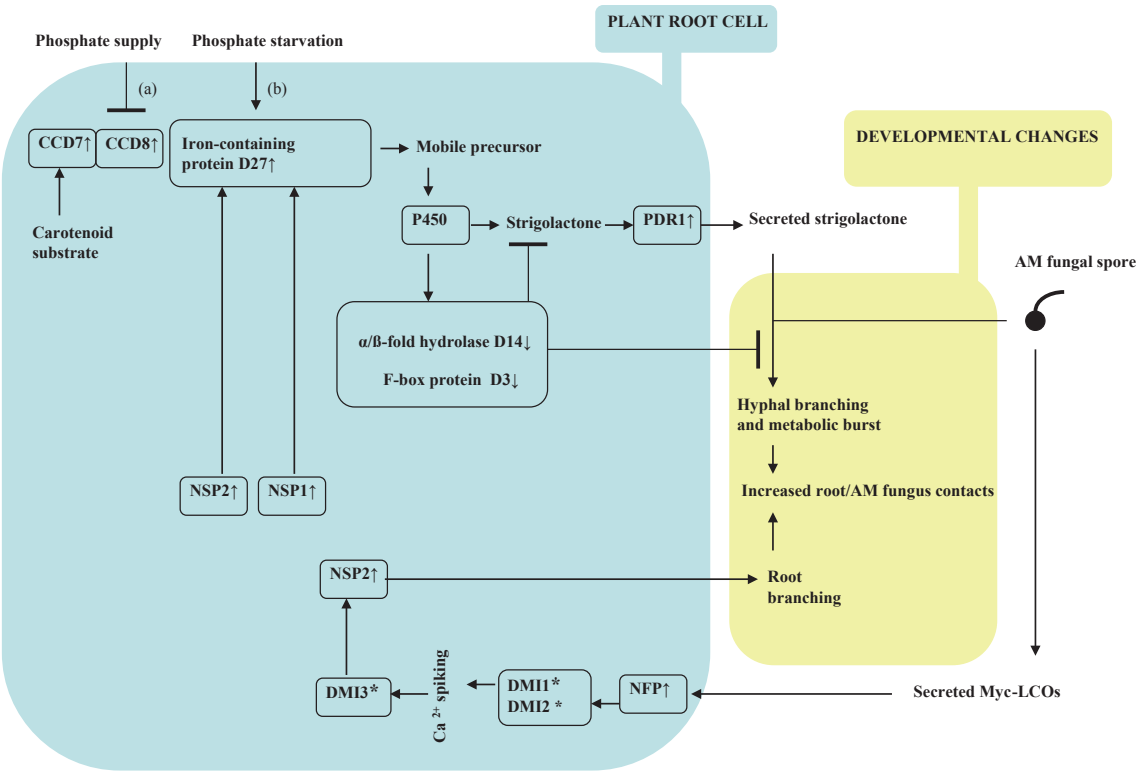

Fig. 2 Schematic overview of the strigolactone (SL) and Myc-LCO-related pathways that mediate arbuscular mycorrhizal (AM) hyphal and root branching responses. Inferred from silencing-based experiments, up- and down-headed arrows indicate plant proteins believed to maximize and minimize plant–fungus contact events, respectively. Because reduced expression of the corresponding genes mostly results into quantitative differences in root colonization without affecting arbuscule morphology, it seems likely that these proteins play roles in AM symbiosis rather through impacting hyphal penetration events than through mediating intra-radical development. Asterisks correspond to plant proteins belonging to the SYM pathway required for fungal colonization. Phosphate regulation events refer to the data provided by: (a) Breuillin *et al.* (2010) and (b) Liu *et al.* (2011).

hyphal penetration events and retarded intraradical expansion, rather than to defects in intracellular development.

A second milestone decisive in understanding the pre-symbiotic crosstalk between mycosymbionts and plants roots was the chemical elucidation of some diffusible AM fungal signalling molecules belonging to the so-called Myc factors. These stimulate root growth and branching through a calcium signal that determines the activation of essential symbiotic genes. It has been shown that the AM fungus *R. irregularis* secretes symbiotic signals corresponding to a mixture of sulphated and nonsulphated simple lipochitooligosaccharides (Myc-LCOs), thus confirming the working hypothesis that Myc signals are ancestors of the more recent LCO-like Nod factors produced by most rhizobia and required for early steps of legume infection and nodule organogenesis (Maillet *et al.*, 2011). Strikingly, studies also revealed that the intricacy of Nod and Myc signalling was more complex than previously anticipated. Initially, the symbiotic signalling pathway identified in *Medicago truncatula* included genes coding for Nod factor perception (*NFP* and *LYK3*), calcium signalling (*DMI1, DMI2* and *DMI3*), and transcription factors (*NSP1 NSP2* and *ERN*). NFP and LYK3 correspond to Nod factor membrane receptors so far described dispensable for AM fungi signal-induced calcium oscillations in epidermal cells and subsequent mycorrhiza formation. The three transcription factors were thought to be specifically activated upon Nod factor perception, while DMI1, 2 and 3 were required both for nodulation and mycorhization (Maillet *et al.*, 2011 and references therein). However, Op den Camp *et al.* (2011) provided evidence that in *Parasponia*, the only nonlegume partner of rhizobia, a single cell surface receptor can recognize both fungal and bacterial signals and induce the common SYM pathway to promote the intracellular accommodation of AM fungi and rhizobia. Actually, silencing of the unique *NFP* orthologue in *Parasponia* was found to impair the formation of both symbioses. Likewise, the data obtained by Maillet *et al.* (2011) suggested that NFP is partly involved in the Myc-signal-elicited root branching response, as inferred from the reduced root branching response observed in *nfp* mutants relative to the wild-type. Additionally, by reason of a 40% lower colonization level than wild-type plants exhibited in the *nsp2* mutant, a NSP2-dependent signalling pathway was found to facilitate mycorrhizal root colonization, thus indicating that the transcriptional activator NSP2 does not function exclusively in rhizobium Nod factor signalling (Maillet *et al.*, 2011). In accordance with this view, recent comparative gene expression studies in symbiotic mutants demonstrated that transcriptional reprogramming by AM fungal LCOs strictly depends on MtNSP and largely requires MtDMI3. It is noteworthy that none of the genes related to arbuscule development were activated by Myc-LCOs, suggesting that the function of Myc-LCOs was

restricted to pre-symbiotic AM stages (Czaja *et al.*, 2012). On the basis of transcript profiling experiments in *nsp1* and *nsp2* knockout mutants, it was also shown that NSP1 and NSP2 are indispensable for strigolactone biosynthesis in *M. truncatula* and rice (Liu *et al.*, 2011). The disturbed SL biosynthesis in *nsp1 nsp2* mutant backgrounds was found to correlate with reduced expression of *D27* that encodes the plastid-located iron-containing protein essential for SL biosynthesis. In contrast to nodulation, none of the components of the Nod factor signalling pathway, not even the kinase CCaMK directly active upstream of NSP1 and NSP2, are required for *D27* expression. It was thus proposed that the NSP1 and NSP2 proteins fulfil dual regulatory functions to control not only downstream targets after rhizobium-induced signalling, but also SL synthesis in nonsymbiotic conditions. With regard to AM fungal infection, the *M. truncatula nsp1 nsp2* double mutant shows a reduction in mycorrhizal root infection but without defects in arbuscule development, suggesting that SLs stimulate root colonization exclusively *ex planta* (Liu *et al.*, 2011). Finally, mutations in *D14* and *D3* that encode a α/β-fold hydrolase and an F-box protein, respectively, resulted in rice plants that are insensitive to SL and produce more SLs than the wild-type owing to the lack of feedback suppression. Consistently, hyphae of AM fungi had additional branches when grown in the vicinity of rice roots of *d3* and *d14* mutants relative to wild-type plants (Yoshida *et al.*, 2012). A dual function for the protein D3 was also proposed by Yoshida and co-workers. Actually, although AM hyphal branching was activated in the vicinity of roots of *d3* mutants, hyphae were often unable to extend beyond the epidermal cell layer and progressive internal fungal growth and arbuscule formation were significantly reduced. Once penetration was achieved, arbuscules were shaped similarly to those of controls. On the contrary, *d14* RNAi rice lines displayed an increased mycorrhizal colonization, probably reflecting higher fungal activities by reason of a higher SL production (Yoshida *et al.*, 2012). Although it remains unclear whether the AM phenotypes observed in *d3* mutants are the result of SL perception/signalling, the physiological role of D3 turns out to be distinct from that of D14.

Overall, it appears from the above-cited data that at least nine plant proteins (NFP, NSP1, NSP2, CCD7, CCD8, D27, PDR1, D3 and D14) were demonstrated as either directly or indirectly involved in the fungal-root branching-induced crosstalk that takes place during pre-symbiosis, as schematized in Fig. 2.

2. Best stage setting: 'Fungal entry: the red carpet'

The pre-symbiotic phase of AM symbiosis ends once a hyphal tip has contacted the root epidermis, following exudate-mediated hyphae and root branching events. Unlike aerial pathogens that have to penetrate the leaf cuticle through the differentiation of an appressorium that generates a glycerol-mediated turgor pressure, glomeromycotan fungi develop hyphododia, which are nonmelanized and nonseptate small swellings structures at the hyphal tip that mediate adhesion to epidermal root cells (Genre *et al.*, 2009; Bonfante & Genre, 2010; Heupel *et al.*, 2010). Recently, Gobatto *et al.* (2012) reported that plants mutated in *RAM1*, which encodes a GRAS-domain transcription factor specifically required for Myc factor signalling, displayed a defect in hyphopodium formation at the root surface and were unable to be colonized by AM fungi. Because *ram1* roots showed a more severe phenotype than plants mutated in components of the symbiosis signalling pathway (*dmi1, dmi2* and *dmi3*), the authors hypothesized that RAM1 functioned downstream of the SYM pathway, but might also act in a manner independent of it. Notably, considering the role played by NSP2 in Myc factor signalling (Maillet *et al.*, 2011), they observed that RAM1 could interact with NSP2, but not with NSP1, suggesting that the SYM pathway might trigger GRAS protein complex formation, with the complex NSP2/RAM1 leading to mycorrhiza-specific responses (Gobatto *et al.*, 2012). Concomitantly, RAM1 was found to regulate the expression of *RAM2* that encodes a glycerol-3-phosphate acyl transferase, which promotes cutin biosynthesis and enhances hyphopodium formation (Wang *et al.*, 2012). Considering first that cutin is synthesized as monomers, laid down at the cell surface in an esterified form, and second, that lipid monomers alone could complement the defect in mycorrhizal perception of the *ram2* roots, Wang and co-workers favoured for cutin monomers a signalling role in promoting hyphopodium formation rather than a structural function.

Beside signalling events occurring at the root surface, a third outstanding breakthrough in understanding the plant AM symbiotic programme relates to the root responses elicited upon hyphopodium formation, among which belongs the formation of a pre-penetration apparatus (PPA) that outlines the route for hyphal growth across the plant cell lumen (Genre *et al.*, 2005). Following a cytoplasmic aggregation, which consists of a cytoskeleton-driven accumulation of organelles including the plant nucleus, at the contacted epidermal site, the host cell develops a transcellular cytoplasmic column, the PPA, whose elongation follows the migration of the plant nucleus toward the inner cell wall facing the root cortex (Fig. 1). When using roots of *M. truncatula* expressing a fluorescent tag for the ER, which were challenged with an AM fungus, a necrotrophic pathogen, a hemibiotrophic pathogen, a noncompatible endomycorrhizal fungus, or abiotic stimuli, a correlation was identified between physical stimulation at the cell surface and nuclear repositioning. It was also observed that cytoplasmic aggregation, a cell response shared by the AM fungi and by the two pathogens, is clearly *DMI3/CCaMK*-dependent, thus extending the role of this gene during the contact phase from symbiotic to pathogenic interactions (Genre *et al.*, 2009). A key conserved component of the common SYM pathway, CCaMK, is currently believed to decode the Ca^{2+} spiking that is activated in the host epidermis during initial recognition of endosymbionts and to trigger appropriate downstream signalling pathways leading to gene transcription in association with LjCYCLOPS/MtIPD3. Recently, it has been reported that distinct Ca^{2+} spiking profiles correlate with specific stages of transcellular apoplastic infection (Sieberer *et al.*, 2012). Low-frequency spiking cells are characterized by nuclear migration to the site of future cell infection associated with cytoplasmic reorganization in the vicinity of the nucleus. By contrast, there is an increase in the frequency of Ca^{2+} spiking just before and during initial cortical cell entry by both bacterial and fungal symbionts, which involves an irreversible cell wall disassembly and *de novo* interface synthesis linked to

membrane invagination. It has been proposed that the protein Vapyrin could mediate Ca^{2+}-mediated membrane and cytoskeleton rearrangements during initial stages of root cell infection by rhizobia and AM fungi (Ercolin & Reinhardt, 2011; Sieberer *et al.*, 2012). Actually, in *M. truncatula*, *Vapyrin* RNAi roots show a high frequency of hyphopodia that attempt but fail to penetrate the epidermal cells (Pumplin *et al.*, 2010). In contrast to common *sym* mutants impaired in initial endosymbiotic signalling (Parniske, 2008), in *Vapyrin* RNAi roots, the fungus attempts to penetrate the cells as exemplified by the hyphal projections existing below hyphopodia. Consequently, Pumplin *et al.* (2010) hypothesized that the signalling process necessary to induce fungal penetration was not affected in *vapyrin* knockdown lines, but that the cellular machinery supporting fungal entry and membrane invagination was impaired. Recently, *MtVapyrin* was reported to be induced upon Myc-LCOs application, suggesting that the encoded protein is accumulated in anticipation of colonization (Czaja *et al.*, 2012). Interestingly, the *Vapyrin* mutant hyphopodia phenotype was also observed in transgenic rice lines upon silencing of the *D3* gene, which results in an increased strigolactone production, suggesting a functional relationship between D3 and Vapyrin that remains to be characterized (Yoshida *et al.*, 2012). A critical role for membrane reorganization for fungal accommodation at the early stage of AM symbiosis was also reported by Kuhn *et al.* (2010) who showed that downregulation of the membrane steroid-binding protein 1 *MtMSBP1* through RNAi led to an aberrant mycorrhizal phenotype characterized by thick and septate hyphopodia with aborted penetration attempts. Because *MtMSBP1* encodes a membrane steroid-binding protein involved in sterol homeostasis, it has been proposed that alteration of lipid metabolism is required to sustain membrane invagination and intracellular accommodation of symbionts (Kuhn *et al.*, 2010).

Aside from cellular remodelling events in response to AM fungus sensing, it was recently shown that silencing of the Rac1 GTPase *MtROP9* triggered the stimulation of early root colonization by the AM fungus *R. irregularis* coupled to an inhibition of reactive oxygen species (ROS) and anti-oxidative compounds production in *M. truncatula* roots (Kiirika *et al.*, 2012). RAC proteins are plant-specific small GTPases that function as molecular switches within elementary signal transduction pathways, including the regulation of ROS generation via activation of plasma membrane-associated NADPH oxidases. It was thus concluded that the oxidative burst that occurs 20 min after AM spore inoculation, somehow concomitantly with the calcium spiking response (Chabaud *et al.*, 2011), did play a role in mounting an early defence barrier against mycorrhizal colonization. Interestingly, the PPA triggered by AM fungi was found to elicit a specific transcriptome response in epidermal cells, including the *DMI3*-dependent upregulation of an *expansin*-like gene having a role in cell wall plasticity, and the downregulation of the defence-related gene *ACRE264* (Siciliano *et al.*, 2007a,b). *ACRE264* is known to encode the protein Avr9/Cf-9 that is required for full resistance to *Cladosporium fulvum* strains expressing the *Avr9* gene. Consequently, the DMI3-mediated suppression of defence-related genes like *ACRE264* after physical contact with the hyphopodium led Siciliano *et al.* (2007b) hypothesize that plant–AM fungus compatibility requires basal defence responses to be kept under control as observed in compatible plant–pathogen interactions.

In this line of reasoning, a fourth significant recent advance in understanding the early steps mediating symbiont accommodation by plant cells was the discovery that AM fungi do use effector proteins to short-circuit the plant defence programme (Kloppholz *et al.*, 2011). Plants are known to have a basal defence system trained to recognize conserved traits of microbial pathogens termed MAMPS (microbial-associated molecular patterns). Recognition of these epitopes by pattern recognition receptors induces MAMP-triggered immunity, a first line of plant defence to prevent further colonization of the host. In return, microbial colonizers have evolved the capacity to deliver effector proteins inside host cells to cope with MAMP-triggered immunity, often through suppression of host defences (de Jonge *et al.*, 2011; Zamioudis & Pieterse, 2012). When investigating whether AM fungi use effector proteins to short-circuit the plant defence programme, Kloppholz *et al.* (2011) showed that *R. irregularis* secreted a protein, SP7, which can cross plant membranes to interact with the defence-related ethylene-responsive factor ERF19 in the plant nucleus to block the ERF19-mediated transcriptional programme, including the expression of target defence proteins. The constitutive expression of *SP7* in roots was reported to lead to higher mycorrhization while reducing the levels of the fungal pathogen *Colletotrichum trifolii*-mediated defence responses, as assessed by the PR10 marker. Noteworthy, *SP7* expression in the rice blast fungus *Magnaporthe oryzae*, a hemibiotrophic pathogen that can infect both leaves and roots of host plants, results in the reduced expression of defence genes encoding PR10 proteins in rice roots and extends the length of the biotrophic phase, delaying the root decay that characterized the necrotrophic phase. Overall, the results obtained by Kloppholz *et al.* (2011) support the view that SP7 acts as a broad spectrum effector to promote the biotrophic phase of a fungus inside a plant. Aside from this report, the use of transformable hemibiotroph fungal pathogens such as *Colletotrichum* spp. and *M. oryzae* whose development share common features with the AM fungal lifecycle (spore, appressoria and/or hyphopodia, haustoria), coupled to comparative analyses of genome sequences from plant-infecting fungi, also succeeded in revealing AM fungal proteins mediating plant cell entry. Tollot *et al.* (2009) demonstrated as essential the transcription factor STE12 in mediating hyphal penetration of leaf surfaces from appressoria and in some cases for subsequent invasive growth by hemibiotrophic plant pathogens. Introduction of *GinSTE*, a *STE12* homologue, isolated from *R. irregularis*, into a noninvasive mutant of *Colletotrichum lindemuthianum* was found to restore penetration and infectivity of the fungal pathogen in *Phaseolus vulgaris* leaves. Likewise, Erl1, a Ras-like GTPase from the rice blast *M. oryzae* was found to be homologous to the mature amino terminal part of the Gin1 protein of *R. irregularis*. Deletion of *ERL1* in *M. oryzae* resulted in delayed appressorium formation, slow growth *in planta* and reduced intracellular colonization without defect in the necrotic ability of the fungus, indicating that ERL1 is required for invasive growth of root tissues. Because the root browning defect of Δ*erl1* strains could be complemented by the AM fungus gene, it was suggested that Erl1 and Gin-N are orthologues and might be involved in the control of polar hyphal

growth *in planta*, thus extending the hypothesis of common genetic features underlying plant colonization strategies among different fungi (Heupel *et al.*, 2010).

Besides the common SYM pathway, the identification of twelve additional proteins involved in the early intracellular accommodation of AM fungi allows us to propose a hypothetical model illustrating the protein pattern associated with AM fungal entry into plant cells at the early stages of symbiosis, as schematized in Fig. 3.

III. Nominees for a leading role

After traversing the epidermis, AM fungal hyphae enter cortical cells via invagination of the plasma membrane (Bonfante & Genre, 2008), where they branch repeatedly to develop a specialized tree-like structure, known as an arbuscule, which is enveloped by an extension of the host plasma membrane, the periarbuscular membrane that separates the fungus from the plant cell cytoplasm. This delineates, as depicted in Fig. 4, the interface compartment, an apoplastic space that surrounds the fungus and mediates nutrient exchange (Bonfante & Genre, 2008). Despite consisting of an extension of the plant plasma membrane, the periarbuscular membrane displays specific protein features, including the presence of the AM symbiosis-specific phosphate transporters MtPT4 and OsPT11 in barrel medic and rice, respectively (Harrison *et al.*, 2002; Kobae & Hata, 2010), the AM-inducible ammonium transporter GmAMT4.1 in soybean (Kobae *et al.*, 2010), STR half-ABC transporters (Zhang *et al.*, 2010; Gutjahr *et al.*, 2012) and VAMP721d/e proteins in *Medicago* (Ivanov *et al.*, 2012). Imaging also revealed that the periarbuscular membrane is composed of at least two distinct specific protein-containing compartments, corresponding to an arbuscule-branch domain where are specifically located MtPT4, OsPT11, GmAMT4, STR transporters and VAMP721s, as opposed to an arbuscule-trunk domain that contains the blue copper-binding protein MtBcp1, which also localizes to the host PM (Fig. 4). This suggests the occurrence of a *de novo* membrane biogenesis process associated with the dichotomous branching of the hyphae (Pumplin & Harrison, 2009; Kobae & Hata, 2010; Kobae *et al.*, 2010; Zhang *et al.*, 2010; Ivanov *et al.*, 2012). Interestingly, the polar targeting of MtPT4 to the periarbuscular membrane is mediated by a transient reorientation of secretion favouring vesicle fusion with the developing periarbuscular membrane rather than with the PM, and a coincident change in the newly synthesized protein cargo entering the secretory system (Pumplin *et al.*, 2012).

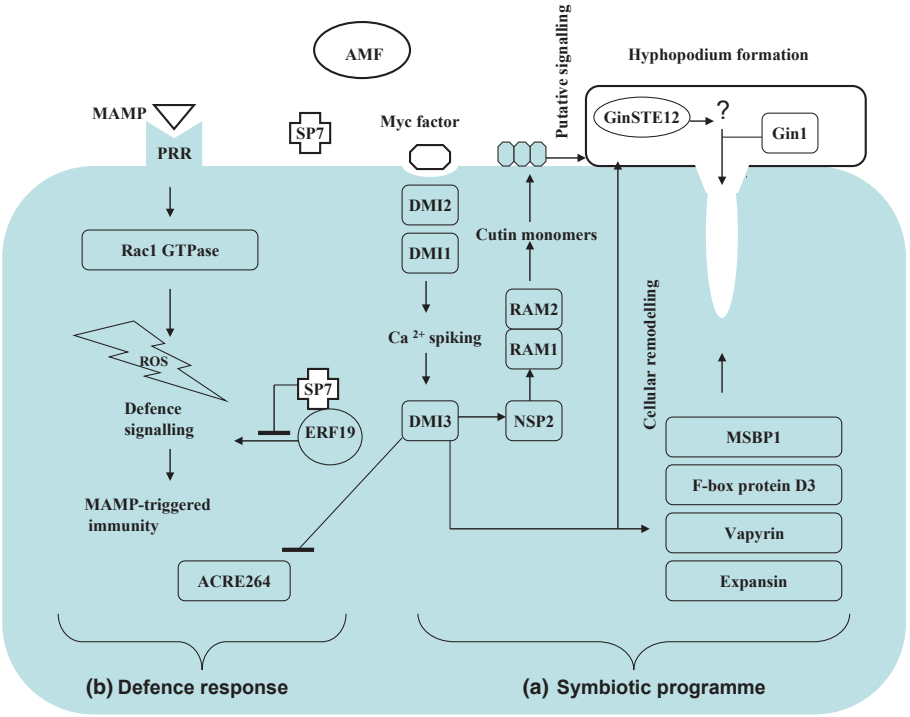

Fig. 3 Schematic representation of a model protein pattern associated with arbuscular mycorrhizal (AM) fungal entry into plant cells at the early stages of symbiosis. (a) The perception by host plants of Myc factors produced by arbuscular mycorrhizal fungi (AMF) induces the symbiotic programme through the SYM pathway (DMI1, DMI2, DMI3) that triggers a NSP2/RAM1/RAM2-mediated signal at the cell surface via the production of cutin monomers, and the activation of cellular remodelling events in both plant (MSP1, Vapyrin, D3, Expansin) and fungal (GinSTE12) partners. (b) Host plants are able to recognize AMF as potential colonizers through pattern-recognition receptors (PRR) that perceive microbe-associated molecular patterns (MAMPs). As a result, a signalling cascade probably involving Rac1-mediated reactive oxygen species (ROS) production (Kiirika *et al.*, 2012) is induced, which results in MAMP-triggered immunity (MTI) through the production of defence-related compounds. In response, AMF have developed the capacity to secrete the SP7 protein effector into the plant cytosol, which upon targeting the nucleus, interacts with the defence-related transcription factor ERF19 to block the ERF19-mediated transcriptional programme. The SYM pathway is also possibly involved in the suppression of MTI, as exemplified by the *DMI3*-dependent downregulation of the defence-related protein ACRE264 (Siciliano *et al.*, 2007a,b). White and blue colours refer to fungal and plant structures/metabolites/proteins, respectively.

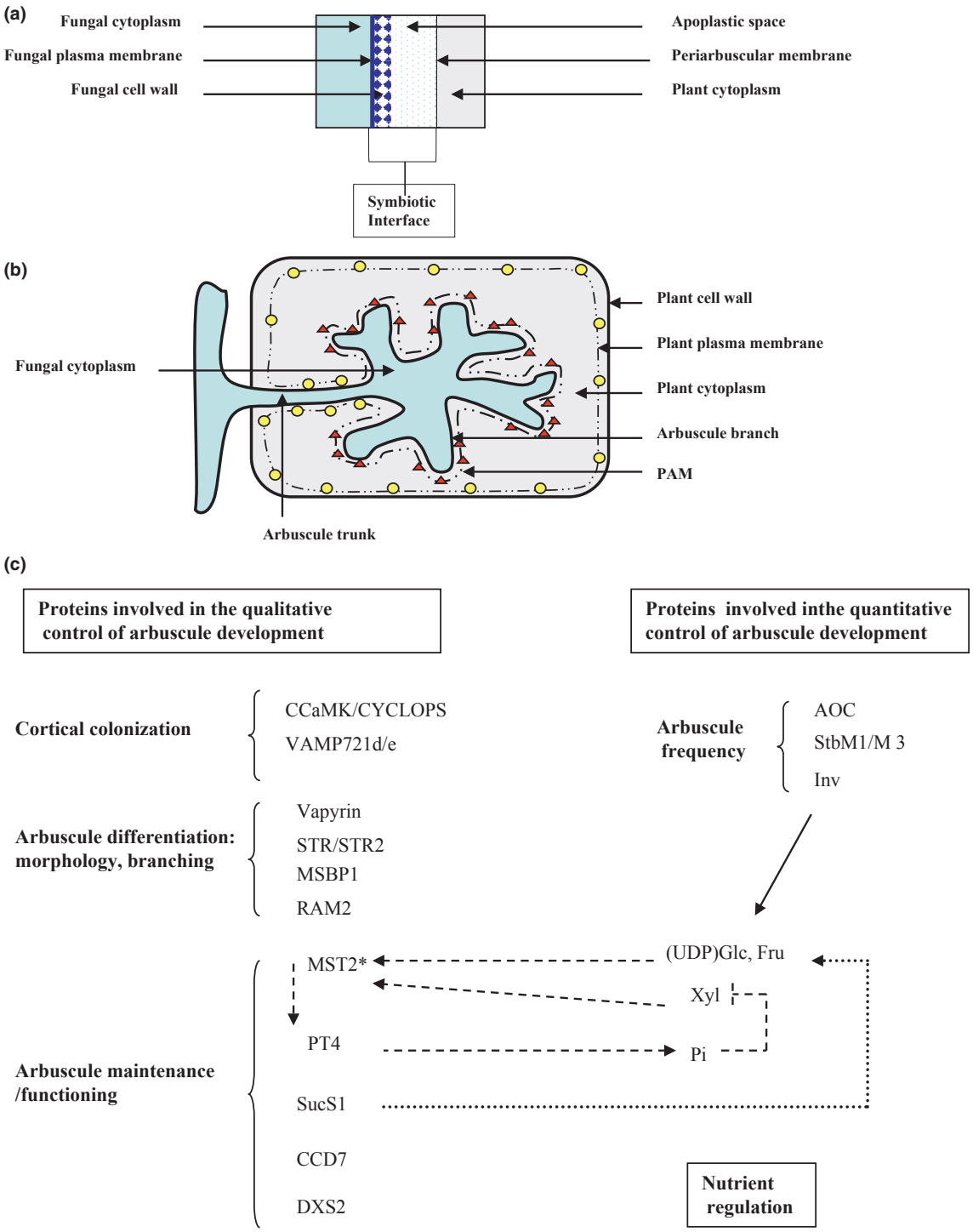

Fig. 4 Schematic representation of the structure and regulation of an arbuscule. (a) Diagrammatic view of the symbiotic arbuscule/cortical cell interface that comprises the plant and the microbial membranes separated by a plant-derived apoplast. (b) Illustration of the polarization of the periarbuscular membrane (PAM) that is composed of at least two distinct specific protein-containing compartments, corresponding to an arbuscule-branch domain that specifically harbours, depending of the host plant, MtPT4, OsPT11, GmAMT4, STR transporters and VAMP721s (red triangles), as opposed to the arbuscule trunk domain that contains the blue copper-binding protein MtBcp1 (yellow circles), which also localizes to the host plasma membrane. This suggests the occurrence of a *de novo* membrane biogenesis process associated with the dichotomous branching of hyphae. (c) Hypothetical model representing the protein- and nutrient-mediated mechanisms involved in the regulation of arbuscule morphogenesis and functioning. Protein roles are inferred from the phenotype(s) displayed in their corresponding loss-of-function backgrounds (Table 1). The regulatory functions of Glc/Fru, Pi and Xyl originate from the data reported in Baier *et al.* (2010), Helber *et al.* (2011) and Schaarschmidt *et al.* (2007), as represented by dot, dashed, and black lines/arrows, respectively. The fungal origin of MST2 is indicated by an asterisk.

Despite this fifth major breakthrough in the understanding of arbuscule morphogenesis, the protein-encoding genes sustaining the cortical phase of AM symbiosis have been a long time coming relative to those involved in hyphal penetration. Nowadays, the blind dissection of the molecular components sustaining arbuscule development/functioning largely benefits from the combination of whole-genome transcriptome profiling of mycorrhizal roots, together with mutant resources and/or reverse genetic screenings for altered mycorrhizal phenotypes (Hirochika *et al.*, 2004; Porceddu *et al.*, 2008; Kuromori *et al.*, 2009; Revalska *et al.*, 2011). Likewise, the ever increasing number of completed plant and fungal genome sequencing projects also has improved the analogy-based targeted search of proteins involved in AM symbiosis (Schmidt & Panstruga, 2011). Overall, the proteins that have so far been demonstrated to play a role in sustaining arbuscule formation/functioning can be divided into three major functional groups, which encompass processes related to membrane biogenesis/protein trafficking, nutrient transport and plastid metabolism.

1. Best film set: 'Membrane biogenesis and protein trafficking'

Aside from resulting in septate hyphopodia with aborted penetration attempts, inactivation of the membrane steroid-binding protein MtMSBP1 also leads to a decreased number of arbuscules, some of them having a distorted morphology, indicating that alteration of sterol metabolism with regard to membrane biogenesis is required for fungal accommodation in cortical cells (Kuhn *et al.*, 2010). As a result, it has been suggested that a common cellular mechanism may be required to sustain hyphal growth through epidermal cells and arbuscule development in the cortex (Pumplin *et al.*, 2010). Sustaining this view, the pattern of impaired epidermal penetration together with lack of arbuscule was also observed in *Vapyrin* knockdown roots in petunia and barrel medic (Feddermann *et al.*, 2010; Pumplin *et al.*, 2010). Actually, when the AM fungus succeeds entering the cortex of *Vapyrin* RNAi plants under high inoculum pressure, intercellular hyhae spread laterally but no arbuscules are formed, a phenotype reminiscent of that displayed in the *cyclops/ipd3* and *pam1* mutants of soybean and petunia (Reddy *et al.*, 2007). Using confocal microscopy, it was shown that the cortical cells of the mutant were indeed colonized, but that arbuscule development was arrested at an early point of branching (Feddermann *et al.*, 2010). By contrast, in wild-type colonized cells, Vapyrin becomes increasingly localized in areas of intense hyphal branching to membrane-bound structures associated with the tonoplast, referred to as tonospheres, which may function as a mobile reservoir of membrane material. This hypothesis could explain why Vapyrin is more critical in cortical cells than in epidermal cells, where there is a more extensive need in membrane production (Pumplin & Harrison, 2009). Likewise, mutation in *RAM2* not only affects hyphopodium formation at the root surface, but also leads to arbuscule defect in the cortex (Wang *et al.*, 2012). It was thus concluded that the cutin monomers generated by RAM2 also act in cortical cells to promote mycorrhizal colonization.

In relation to protein trafficking, Takeda *et al.* (2009) investigated in *Lotus japonicus* the relevance for mycorrhizal development of two AM-specific subtilases, SbtM1 and SbtM3, by negatively interfering with their expression through RNAi. Suppression of *SbtM1* or *SbtM3* caused a decrease in intra-radical hyphae and arbuscule frequency without affecting the number of fungal penetration attempts, indicating that the two subtilases play an indispensable role during the fungal infection process, in particular arbuscule development. The predicted proteolytic activity of SbtM1 and SbtM3, together with their localization in the apoplastic perifungal and periarbuscular spaces, suggested that cleavage of structural proteins within and between plant cell walls by subtilases might be required for elongation of fungal hyphae in the intracellular space and for penetration into the host cell during arbuscule formation. Very recently, Ivanov *et al.* (2012) analysed whether an exocytotic pathway might similarly control the formation of the symbiotic interface in both RNF and AM symbioses. Exocytosis that involves fusion of transport vesicles (with a specific cargo) with their target (plasma) membrane, is mediated in plants by a group of proteins belonging to the VAMP72 (vesicle-associated membrane proteins), which have been shown to be recruited in the Arabidopsis interaction with biotrophic fungi (Kwon *et al.*, 2008). Upon mining of *Medicago* EST and genome sequence data, a putative 'symbiotic' *VAMP721* subgroup, including *MtVAMP721d* and *MtVAMP721e* without *Arabidopsis* homologues, was retrieved as the best candidate to be involved in symbiosis-related membrane compartments. Localization studies showed that the corresponding proteins accumulate over the periarbuscular membrane, especially at the fine branches, and subsequent RNAi-based silencing of *VAMP721d/e* genes blocked symbiosome as well as arbuscule formation in RNF and AM symbiosis, respectively. It was thus concluded that arbuscule formation is specifically controlled by the MtVAMP721d/e-regulated exocytotic pathway whose switch-on allows the targeting of vesicles with a different cargo to facilitate the development of a symbiotic interface with specific protein composition.

Also related to the processes that may drive arbuscule extension/differentiation, Zhang *et al.* (2010) identified from a mycorrhiza-defective *Medicago* mutant background two half-ABC transporters designated STR (for stunted arbuscule), which turned out to be essential for arbuscule development but not indispensable for RNF symbiosis. Expression of both *STR* genes was induced in cortical cells containing arbuscules and silencing of *STR2* by RNAi resulted in a stunted arbuscule phenotype identical to that of *str*. Interaction data showed that STR and STR2 function as a heterodimer that resides in the periarbuscular membrane, more specifically around arbuscule branches. The authors supposed that unlike the phosphate transporters MtPT4 and OsPT11, the STR/STR2 dimer acts as a pump to export a substrate molecule, which might be a lipid or a secondary metabolite, from the cortical cells to the periarbuscular apoplastic space in direction to the AM fungus. By reason of the slow growth and reduced branching phenotype of arbuscules typical of *str* phenotypes, it was proposed that strigolactones might play a role in stimulating the dichotomous branching of intra-radical hyphae to create the arbuscule (Zhang *et al.*, 2010). However, in a parallel study that analysed the function of STR/

Table 1 Repertoire of proteins listed in the current study for which partial or total loss-of-function supports a role in sustaining intra-radical and/or arbuscule (Arb) development and/or functioning during arbuscular mycorrhizal (AM) fungal symbiosis

Protein	Origin	Function	Location	Origin of loss-of function	Loss-of-function phenotype in AM symbiosis	Main literature cited
CCaMK/CYCLOPS	Plant	Signalling	Plant nucleus	Nodulation defective mutant	Arb defective	Reviewed in Singh & Parniske (2012)
Vapyrin	Plant	Membrane biogenesis	Membrane-bound structures	RNAi	Arb branching defective	Feddermann *et al.* (2010); Pumplin *et al.* (2010)
MSBP1	Plant	Membrane biogenesis	ER	RNAi	Decreased arb number, arb morphology defects	Kuhn *et al.* (2010)
SbtM1/M3	Plant	Protein trafficking	Apoplastic symbiotic interface	RNAi	Decreased IRM and arb number	Takeda *et al.* (2009)
VAMP72s	Plant	Membrane biogenesis	PAM branches	RNAi	Arb defective	Ivanov *et al.* (2012)
RAM2	Plant	Cutin biosynthesis	Root surface and cortex	Mycorrhization defective mutant	Arb defective	Wang *et al.* (2012)
STR/STR2	Plant	Transport	PAM branches	RNAi	Slow growth, reduced branching	Zhang *et al.* (2010); Gutjahr *et al.* (2012)
PT4	Plant	Transport	PAM branches	RNAi	Arb premature death, growth arrest	Javot *et al.* (2007)
Inv	Plant	Suc cleavage	Apoplast	Activity inhibitor	Reduced mycorrhization	Schaarschmidt *et al.* (2007)
SucS1	Plant	Suc cleavage	Cytoplasm	RNAi	Reduced IRM and vesicles. Arb branching defects and senescence	Baier *et al.* (2010)
MST2	Fungus	Transport	Arbuscule/ IRM/ERM	HIGS	Reduced mycorrhization, not fully developed arb, arb senescence	Helber *et al.* (2011)
DXS2	Plant	Carotenoid biosynthesis	Plastid	RNAi	Arb senescence	Floss *et al.* (2008)
CDD7	Plant	Carotenoid biosynthesis	Plastid	RNAi	Reduced mycorrhization, arb senescence	Vogel *et al.* (2010)
AOC	Plant	JA biosynthesis	Plastid	RNAi	Reduced mycorrhization	Isayenkov *et al.* (2005)

ER, Endoplasmic reticulum; ERM, extra-radical mycelium; HIGS, host-induced gene silencing; IRM, intra-radical mycelium; JA, jasmonic acid; PAM, periarbuscular membrane; RNAi, RNA interference; Suc, sucrose.

STR2 heterologous proteins in rice, this hypothesis was discarded in so far as *d17* and *d10* rice lines that carry mutations in carotenoid cleavage dioxygenases 7 and 8, respectively, which are required for SL biosynthesis, displayed wild-type like branched arbuscules (Gutjahr *et al.*, 2012). Consequently, the involvement of lipid and/or cutin-related compounds as alternative substrates of the STR transporter complex has been suggested (Wang *et al.*, 2012).

2. Best character part: 'On a permanent slimming diet, the phosphate price for success'

As a central feature of biotrophic mutualism during AM symbiosis, a bi-directional nutrient flux corresponding to carbon delivery to the fungus vs mineral nutrient import to the plant, has been for a long time anticipated to proceed through protein transporters located at the arbuscule/cortical cell interface. Regarding P, which remains in consideration as the main symbiosis-mediated benefit for the host owing to the low mobility of its ionic forms in the soil, two pathways can mediate its acquisition by plants as orthophosphate (Pi): the direct uptake pathway at the root–soil interface that involves high-affinity Pi transporters located in the epidermis and root hairs, and the mycorrhizal pathway that extends from extra-radical hyphae to cortical arbuscules. Pi is believed to translocate to fungal hyphae as polyphosphate, which upon hydrolysis by polyphosphatases in the arbuscule, generates Pi that is exported to the periarbuscular space. Subsequently, plant Pi transporters (PT), which use an H^+ gradient to drive the transport process (Gianinazzi-Pearson *et al.*, 2000), mediate Pi import to the root cell across the periarbuscular membrane (Parniske, 2008). Currently, AM-inducible Pi transporters, which all belong to the clade Pht1, have been identified in many plant species and cluster in subfamilies I and III (Javot *et al.*, 2007). Transporters in subfamily III are expressed in roots, and some members such as StPT3 of potato and LjPT3 of *L. japonicus* are induced in cortical cells during AM symbiosis. Noteworthy, in *L. japonicus* roots colonized with *Glomus mosseae*, *LjPT3* transcripts were detected in arbuscule-containing cells of the inner cortex and partial suppression of *LjPT3* through RNAi led to a two-fold reduction in Pi transfer coupled to a decreased arbuscule number. This suggests that LjPT3 actually contributes to the symbiotic transport of Pi and that insufficient Pi

uptake and/or low expression of *LjPT3* prevents further development of fungal structures (Maeda *et al.*, 2006). Relative to subfamily III, Pi transporters belonging to subfamily I such as OsPT11 of rice, MtPT4 of *M. truncatula* and LePT4 of tomato, only accumulate during symbiosis within arbuscule-containing cells, and at least for MtPT4 and OsPT11, their location in the periarbuscular membrane, notably in the arbuscule-branch domain, has been demonstrated (Pumplin & Harrison, 2009; Kobae & Hata, 2010), and thereby they are also referred to as mycorrhiza-specific transporters (Javot *et al.*, 2007).

The functional demonstration for a role of subfamily I transporters in sustaining symbiotic Pi transport comes from *mtpt4* knockout mutants in *M. truncatula*, which fail to display mycorrhiza-associated increases in Pi (Javot *et al.*, 2007). Likely as a sixth decisive breakthrough in deciphering mycorrhiza functioning, Pi transport by MtPT4 appears clearly essential for AM symbiosis as inferred from the premature death of arbuscules and fungal growth arrest in mutants lacking MtPT4 function, suggesting that the import of Pi by MtPT4 serves as a signal to the plant cell not only to permit continued arbuscule development, but also to sustain fungal existence within plant roots (Javot *et al.*, 2007). Consistent with the idea of a causal relationship between Pi transport and arbuscule maintenance, proteins MtPT4 and OsPT11:GFP were no longer detectable on degenerating arbuscules in wild-type roots of *M. truncatula* and rice, respectively (Harrison *et al.*, 2002; Kobae & Hata, 2010). Likewise, in petunia plants it was noticed that repression of phosphate transporter-encoding genes upon Pi addition preceded the reduction in colonization, indicating that PT loss-of-function actually represents a cause, but not a consequence, of decreased symbiosis (Breuillin *et al.*, 2010). Unexpectedly, the N status of the plant was found to impact the *mpt4* mycorrhizal phenotype in so far as premature arbuscule degeneration is relieved when plants are deprived of N, whereas fungal death is not rescued when the fungus has access to carbon from a nurse plant, indicating that arbuscule lifespan is regulated in part by N, but unlikely by C availability (Javot *et al.*, 2011). Noteworthy, AM symbionts are known to transfer N to the plant (Hodge *et al.*, 2001) and the existence of AM-inducible ammonium transporter genes has been documented especially in *L. japonicus* and *Glycine max* (Guether *et al.*, 2009; Kobae *et al.*, 2010). It is therefore conceivable that ammonium delivery by the AM fungus at the cortical plant–symbiont interface can also act as a signal to sustain arbuscule functioning.

3. Best visual effects: 'Feeding on carbon'

Regarding plant carbon delivery to the AM fungus, sucrose (Suc), which represents a substantial portion of the photosynthetic fixed CO_2, is used for long-distance carbon and energy transport into diverse heterotrophic sinks and consists of the preferred carbohydrate translocated to the mycorrhizal interface (Bucking & Shachar-Hill, 2005). Because intra-radical fungal structures are unable to take-up Suc, and owing to the lack of evidence in favour of Suc-cleaving activities in AM fungi, Suc is believed to be hydrolysed before fungal utilization by cytosolic Suc synthases (SucS), producing UDP-Glc and Fru, or by invertases (Inv), producing Glc and Fru. Particularly, extracellular invertases have a key function in supporting increasing sink strength, a feature of mycorrhizal roots, and may thus directly deliver utilizable carbohydrates to the apoplast-located fungal structures. Unexpectedly, artificially augmented hexose availability to the AM fungus, obtained by a yeast-derived apoplastic Inv active in the arbuscule interface of transgenic mycorrhizal *M. truncatula* roots, failed to improve AM colonization significantly (Schaarschmidt *et al.*, 2007). These data suggested that carbohydrate supply in AM cannot be improved by root-specifically increased hexose concentrations, implying that under normal conditions sufficient carbon is available in mycorrhizal roots. By contrast, transgenic tobacco plants expressing an inhibitor of Inv functioning showed reduced apoplastic invertase activities in roots that also had lower contents of Glc and Fru coupled to a diminished mycorrhization. Consequently, the carbon supply in the AM interaction actually depends on the activity of hexose-delivering apoplastic invertases in roots (Schaarschmidt *et al.*, 2007). Concomitantly, to study the relevance of the SucS-mediated symplastic sink near the plant fungus interface, Baier *et al.* (2010) used *M. truncatula* lines displaying partial suppression of *MtSucS1*, the only *MtSucS* gene currently known to be activated under endosymbiotic conditions (Baier *et al.*, 2007). Silencing of *MtSuc1* led to an internal mycorrhization-defective phenotype as inferred from reduced frequencies in internal hyphae, vesicle and arbuscule development. Strikingly, arbuscules were not only degenerating, but often showed a lower branching network, resulting in a reduced functional symbiotic interface also evident from the recorded down-regulation of periarbuscular membrane transcript markers, including the phosphate transporter gene *MtPT4*. This phenotype, somehow reminiscent of that displayed in knockdown *MtMTP4* constructs (Javot *et al.*, 2007), correlated with reduced P and N concentrations and was proportional to the extent of MtSuc1 knockdown. Overall, it was concluded that plant sucrose synthase MtSuc1 functioning is directly or indirectly a prerequisite, not to induce, but to sustain normal arbuscule maturation and lifetime (Baier *et al.*, 2010).

A recent seventh likely decisive step forward in understanding mycorrhizal mutualism comes from the first isolation of a *R. irregularis* monosaccharide transporter operating at several symbiotic root locations (Helber *et al.*, 2011). Using a preliminary draft of the sequencing project of *R. irregularis*, Helber and co-workers identified the monosaccharide transporter gene *MST2*, which is expressed not only in arbuscules but also in intercellular hyphae, indicating that sugar uptake can proceed in both fungal structures. MST2 was found to be able to transport Glc, and Fru, but also Xyl, Man, Gal, glucuronic and galacturonic acids that are components of the cell wall-like apoplastic plant-fungus interface, thus corroborating the idea that AM fungi can indeed feed on cell wall components (Smith & Smith, 1990). Furthermore, *MST2* expression *in planta* was clearly found to depend on the symbiotic phosphate delivery pathway. Actually, upon Pi fertilization, the expression of *MST2* was downregulated concomitantly with that of the mycorrhiza-specific Pi transporter *PT4*. Knockdown *MST2* lines through host-induced gene silencing indicate that MST2 is indispensable for a functional symbiosis as inferred from lower mycorhization levels coupled to the appearance of not fully

developed and early senescing arbuscules, a phenotype that parallels the abolished expression of *MtPT4*. Unexpectedly, it also turned out that Xyl can specifically induce the expression of *MST2* in the extra-radical mycelium, a tissue preliminarily believed to be incapable of sugar uptake, suggesting that Xyl may act as a signal to trigger *MST2* expression *in planta*. On the basis of these data, Helber *et al.* (2011) proposed a model according to which AM fungal growth within the cortex induces a signal-mediated increase in carbon sink coupled to Xyl availability that triggers *MTS2* expression, and subsequent arbuscule formation induces *PT4* activation. As a feedback control, a high phosphate symbiotic delivery might act by reducing Xyl concentrations and consequently repressing *MST2* induction (Fig. 4).

4. Best makeup: 'Plastid-derived metabolites'

Upon AM symbiosis, two types of apocarotenoids (carotenoid cleavage products) are synthesized in the plastids that proliferate around arbuscules, which lead to the typical macroscopically visible yellow coloration of many mycorrhizal roots (Strack & Fester, 2006). Of unknown function, these compounds that further accumulate in the cytosol and/or vacuoles, are assumed to originate from a common carotenoid precursor (Fester *et al.*, 2002; Floss *et al.*, 2008). The chromophor of this yellow complex is an acyclic C_{14} apocarotenoid polyene called mycorradicin that occurs in a complex mixture of derivatives, coupled to the concomitant accumulation of C_{13} cyclohexanone apocarotenoid derivatives (Schliemann *et al.*, 2006). To investigate the elusive role of cyclohexanone and mycorradicin accumulation in AM symbiosis, Floss *et al.* (2008) suppressed the expression of *MtDXS2* (1-deoxy-D-ribulose 5-phosphate synthase) that catalyses the first step of the plastidial methylerythritol phosphate (MEP) pathway, which supplies isoprenoid precursors in parallel to an alternative cytosolic pathway. RNAi-mediated repression of *MtDXS2* led to a strong and reproducible reduction in the accumulation of the two AM-inducible apocarotenoids coupled to a shift towards a greater number of older, degrading and dead arbuscules at the expense of mature ones. Overall, these data reveal a requirement for DXS2-dependent MEP pathway-based isoprenoid products to sustain mycorrhizal functionality at late stages of symbiosis. In accordance with this view, Vogel *et al.* (2010) reported that a knockdown approach performed in tomato on the *carotenoid cleavage dioxygenase 7* (*cdd7*) gene located downstream in the pathway of apocarotenoid biosynthesis resulted in major decreases in the concentrations of AM-induced apocarotenoids in *cdd7* antisense lines, coupled to a reduced arbuscule abundance. It is noteworthy that a high exogenous supply of Pi in mycorrhizal petunia roots was found to repress genes involved not only in phosphate transport and intracellular accommodation, but also in carotenoid biosynthesis (Breuillin *et al.*, 2010). Taken together, these data support a hypothesis according to which apocarotenoids sustain directly or indirectly arbuscule maintenance/functioning.

Finally, and as very comprehensively reviewed in Hause & Schaarschmidt (2009), the involvement of the plastid-located lipid-derived phytohormone jasmonic acid (JA) as a regulator of AM symbiosis has been observed in diverse plant species. Initially noticed from application experiments that resulted in a promotion of mycorrhizal root colonization, a positive effect of JA on AM symbiotic development was also inferred from the increased endogenous JA concentrations observed after the initial step of the symbiotic interaction. A functional demonstration for a role of JA in mycorrhiza establishment comes from a *M. truncatula* antisense line that displayed a partial suppressed expression of *MtAOC1*, which encodes the JA-biosynthetic enzyme allene oxide synthase (AOC) (Isayenkov *et al.*, 2005). The reduction in the amount of MtAOC protein through antisense-mediated suppression resulted in a decrease in endogenous JA concentration in mycorrhizal roots, accompanied by an overall reduction in arbuscule frequency rather than to an abnormal infection process. Immunocytology indicated that in mycorrhizal roots MtAOC clearly localizes to plastids that develop around arbuscules, whereas the cortex cells of nonmycorrhizal roots were label-free. Notably, the AOC protein also seems to be present in arbuscule-containing cells independent of their developmental stage, suggesting the absence of a relationship between JA synthesis and arbuscule maintenance. When considering genes the repression of which upon Pi fertilization may potentially affect AM colonization in petunia, a homologue of the JA-inducible *JA2* transcript was slightly increased, in contrast to the phosphate-mediated suppression of those essential for symbiosis (Breuillin *et al.*, 2010). Consequently, the petunia transcriptional Pi-related proxy sustains a helper/signalling effect of JA biosynthesis in mycorrhizal colonization rather than a prominent role of the phytohormone in sustaining AM functioning.

Overall, contrasting with the last decade that has been essentially dominated by the dissection of the role played by the common SYM pathway in the cortical infection by AM fungi (reviewed in Singh & Parniske, 2012), recent years have seen not only an extraordinary increase in the protein repertoire sustaining arbuscule development and functioning, but also an unexpected specialization of the protein machinery mediating arbuscule morphogenesis coupled to a network of interacting regulators that turns out to be quite more complex than anticipated, a scenario we have attempted to schematize and list in Fig. 4 and Table 1, respectively.

IV. Future artists

In recent years, the expansion of legume genome data banks coupled to imaging and gene-to phenotype reverse genetic tools such as RNAi, have emerged as very efficient methods to elucidate plant-related protein mechanisms sustaining AM symbiosis development and functioning. Although expected, but likely to a lesser extent, most of the resulting data support a drastic role of membrane organogenesis and differentiation in accommodating AM fungal symbionts, through which C, Pi and N turned out to play decisive roles. Likewise, despite the multinucleate nature of AM fungi and the recurrent absence of transformation tools, host-induced gene silencing succeeded for the first time in switching off fungal gene expression *in planta*, thus unlocking a methodological bottleneck. In this respect, methodological refinements and future insights will surely improve our knowledge about AM fungal dependence upon plant and soil metabolites, as demonstrated

above for Xyl. Likewise, symbiosis with AM beneficial fungi is known to promote plant fitness and help hosts to cope with environmental stresses, a phenomenon somehow anticipated, on the basis of previous results obtained with cadmium tolerance of mycorrhizal plants (Aloui *et al.*, 2009, 2011), to depend upon the constitutive symbiotic protein programme. Consequently, one can expect that future silencing-based approaches may not only increase knowledge about the functions essential to the AM symbiotic partnership, but also shed light on the processes by which mycorrhiza can help plants to face adverse environments.

Acknowledgements

C.A., E.D.G., D.W. and G.R. acknowledge financial support by the Burgundy Regional Council (PARI Agrale 8) and 'Fonds National de la Recherche du Luxembourg' AFR TR-PHD BFR 08-078. The authors are grateful to J. Negrel, B. Schoefs, H-N. Truong-Cellier and D. van Tuinen for their critical readings of the review.

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