Signaling and Communication in Plants

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Signaling and Communication in Plant Symbiosis

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Preface

Because of their photosynthesis, plants are the major primary producers in terrestrial ecosystems and a precious source of organic carbon for all their microbial symbionts. Biotrophic microbial symbionst derive nutrients from the tissues of the living host, and they often colonise the plant cells where they form intracellular structures that are the site of nutrient uptake and exchange, as well as of interorganismal signaling and communication. To colonise the plant tissues via balanced endosymbiosis, all biotrophic microbes, irrespective of their trophic strategy, need to overcome the plant defense responses through an exchange of molecular signals.

Plants are unique as they are able to associate with both prokaryotic and eukaryotic microbes and establish with them well-balanced symbiotic interactions that range from mutualism to antagonism. No other multicellular organisms give rise to this variety of symbiotic interactions. Plants must be able to discriminate between mutualistic micro-organisms that may exchange organic carbon for essential nutrients such as nitrogen and phosphorus, thus promoting plant growth, and antagonistic pathogens that are only detrimental and cause disease. As this volume is making clear, a fine tuning of the signals in plant symbioses is very complex and still only partially understood.

This volume provides overviews of the current knowledge on a variety of symbiotic systems. The first section is dedicated to signalling during the formation of mutualistic symbioses in legume plants. Legume plants have been pivotal to understand the genetic bases of symbioses and the comparison between nodule and arbuscular mycorrhizal (AM) symbioses has revealed a common symbiosis (SYM) signalling pathway leading to intracellular accommodation of fungal and nitrogen fixing bacterial endosymbionts. The recent discovery that AM fungi secrete symbiotic signals that resemble rhizobial lipochito-oligosaccharides (Maillet et al. 2011) brings the similarities between these two symbioses even further. Despite the importance of legumes as model systems to study mutualistic symbioses, there is a great diversity of plant-microbe interactions that involve nitrogen fixing bacteria others than rhizobia, and a variety of other mycorrhizal and endophytic fungi. Some of the chapters in this book provide current knowledge on these diverse

interactions, and witness the progress in the unravelling of genetic determinants in plant-microbe signalling, and the impressive amount of data emerged from the use of both genomic and post-genomic approaches. The last section of the book is focused on the interaction of plants with antagonistic biotrophs, ranging from filamentous fungi to oomycetes, and to nematodes.

Torino — Silvia Perotto
Bonn — Frantisek Baluska

Reference

Maillet et al. 2011. Fungal lipochitooligosaccharides symbiotic signals in arbuscular mycorrhiza. Nature, 469:58–63

Contents

The Role of Diffusible Signals in the Establishment of Rhizobial and Mycorrhizal Symbioses

J. Benjamin Miller and Giles E.D. Oldroyd

Abstract The roots of at least 80% of all angiosperms are able to engage in symbiotic relationships with arbuscular mycorrhizal (AM) fungi of the group Glomeromycota in order to derive macro- and micro-nutrients from the environment (Brachmann and Parniske, PLoS Biol 4:e239, 2006). Legume roots also form a unique symbiosis with rhizobia in order to derive fixed nitrogen. The establishment of both of these symbioses depends upon signalling between the plant host and the microorganism, of which a number of diffusible signals are essential. Here we discuss the synthesis and role of these diffusible signals for the establishment of both rhizobial and mycorrhizal symbioses.

1 Introduction: A Molecular Dialogue Between Host Plant and Symbiont

In order for symbioses to be established between host and symbiont it is necessary that tightly regulated communication occurs. In the case of symbiotic interactions between plant roots and microorganisms in the rhizosphere, the plant must attract and promote the symbiotic partner to interact with its root, whilst in turn the microorganism must respond to distinguish itself as symbiotic rather than pathogenic and subsequently gain regulated entry into the root. This results in a situation where each organism is required to participate in an elaborate communication in order to allow the establishment and progression of symbiosis. The term "molecular dialogue" was originally coined to describe this communication which occurs between the roots of legumes and rhizobia (Denarie et al. 1993), and is also suitable when considering the interaction between mycorrhiza and host plants.

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2 Diffusible Signals During Nodulation

During the establishment of the legume-*Rhizobium* symbiosis, plant roots release flavonoids which are perceived by rhizobial bacteria. This perception leads to the induction of rhizobial *nod* genes which encode proteins required for the synthesis of Nodulation (Nod) factors. Nod factors are secreted by rhizobia and upon their detection act as signalling molecules to the host plant (Oldroyd and Downie 2006, 2008). Nod factors are essential for nodulation and are important mediators of host-range specificity.

2.1 Flavonoids

The earliest component of the molecular dialogue between legumes and rhizobia is the synthesis of flavonoids by the plant root. These are constitutively produced polyaromatic compounds that are perceived by rhizobia via the NodD protein (Peck et al. 2006). This LysR-type transcriptional regulator then promotes Nod factor biosynthesis by activating the transcription of *nod* genes (Fisher and Long 1993).

2.1.1 Structure and Synthesis

Flavonoids are diverse polyaromatic secondary metabolites consisting of a 15-carbon skeleton and are formed from a branch of the phenylpropanoid pathway. The first committed step of flavonoid biosynthesis is catalysed by chalcone synthase; this reaction involves the condensation of *4*-coumaroyl-CoA with three molecules of malonyl-CoA to form a chalcone flavonoid precursor (Fig. 1). This precursor feeds into further biosynthetic reactions which yield either *5*-deoxyflavonoids or *5*-hydroxyflavonoids. Flavonoids can be further sub-classed into flavonoids and isoflavonoids according to whether the phenyl group is attached to C2 or C3 (as with the flavonol kaempferol or the isoflavone daidzein, respectively; Fig. 1).

Flavonoid production is ubiquitous in plants and these compounds are typically associated with plant defence responses, in addition to lignin and anthocyanin production (Winkel-Shirley 2001). The first proof for the role of root-exuded flavonoids during symbiosis was the induced expression of *Sinorhizobium meliloti* nodulation genes (*nodABC*) by luteolin, a flavone (Peters et al. 1986), and the induction of *Rhizobium trifolii nod* genes by flavones from clover (Redmond et al. 1986). Subsequent research has identified several other flavonoids involved in the nodulation signalling of many plant species (reviewed by Broughton et al. 2000). It has been noted that some flavonoid structures are only produced by particular plants; for example, isoflavonoid production is limited to the Papilionoideae (or Faboideae) subfamily of the Leguminosae (Dixon et al. 2002). This diversity of flavonoid production has been associated with determining, at least in part, the

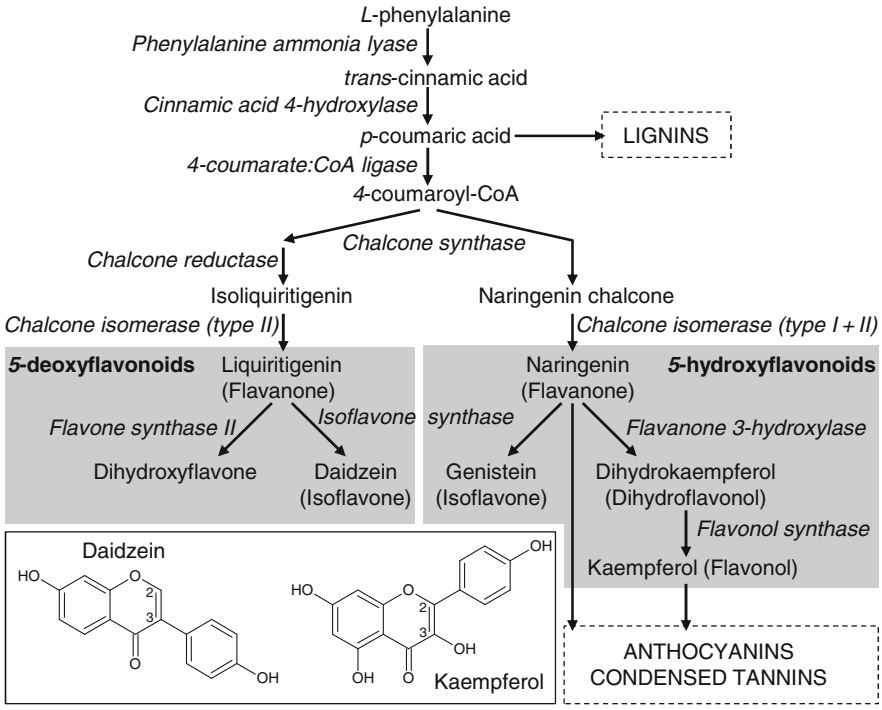


Fig. 1 Biosynthesis of flavonoids. Partial diagram of the phenylpropanoid pathway. Enzymes are depicted in italics; *5*-deoxyflavonoids and *5*-hydroxyflavonoids are denoted by shaded boxes; compounds in dashed boxes represent major side branches of the pathway. Inset shows chemical structures of two example flavonoids isolated from root exudates. Figure adapted from Shaw et al. (2006), Subramanian et al. (2007), Winkel-Shirley (2001) and Zhang et al. (2009)

specificity of *Rhizobium* responses (Cooper 2007; Gibson et al. 2008); for instance, some flavonoids will act as *nod* gene inducers in one context, but as repressors in another (Firmin et al. 1986).

2.1.2 Biological Activity

Two distinct roles for flavonoids and their related molecules have been suggested in the context of early communication during the legume-*Rhizobium* symbiosis: (1) they are involved in chemotaxis responses of rhizobia; (2) they modulate *nod* gene expression of rhizobia (Shaw et al. 2006).

It is essential that rhizobia present in the rhizosphere are attracted to host roots in order for the symbiosis to be formed. Plant roots secrete many compounds into the rhizosphere and in doing so modulate microorganism populations (Dennis et al. 2010). Indeed, it has been estimated that 27% of carbon allocated to roots is

deposited in the rhizosphere (Jones et al. 2009). Legumes must therefore selectively encourage the chemotaxis of rhizobia and flavonoid secretion is important in this process. Positive chemotaxis of *S. meliloti* was specifically demonstrated with the flavone luteolin which is produced by the *S. meliloti* host *Medicago sativa*; this response did not occur with naringenin or apigenin, two closely related flavonoids not produced by *M. sativa* (Caetano-Anolles et al. 1988). Additionally, flavonoid production in host roots is increased upon the addition of compatible *Rhizobium* species (Schmidt et al. 1994). This increased flavonoid synthesis was dependent upon Nod factor structure, implying a positive feedback loop in the regulation of flavonoid production specifically in legume–rhizobia interactions which are symbiotically favourable.

Arguably the most important and most well-characterised rhizobial response to flavonoids is the induction of *nod* gene expression. At least 30 *nod* gene inducing flavonoids have been identified, and their activity is typically in the low micromolar to nanomolar range (Cooper 2004). The exact mechanism by which flavonoids are perceived in rhizobia is unclear; however, the importance of NodD proteins is apparent. NodDs are transcriptional regulators of the LysR-type family and primarily control the expression of *nod* genes, which are responsible for Nod factor biosynthesis (Sect. 2.2). Rhizobia usually contain one to five NodD homologues, depending on species; for example, *S. meliloti* contains three NodDs which share greater than 77% amino acid identity (Honma and Ausubel 1987; Peck et al. 2006). NodDs bind to conserved 55 bp DNA sequences of the promoter region of inducible *nod* genes, the so-called *nod* box, and in doing so induce a bend in the DNA (Fisher and Long 1993). This DNA bending appears to sharpen upon appropriate flavonoid treatment, resulting in subsequent RNA polymerase binding and thereby activating transcription (Chen et al. 2005).

Much genetic evidence suggests that NodD is involved in flavonoid perception. NodD is necessary and sufficient for *nodC* expression in the presence of flavonoids (Mulligan and Long 1985). NodD from *Rhizobium leguminosarum* bv. *viciae* localises to the cytoplasmic membrane (Schlaman et al. 1989), which is also where the flavonoid inducer naringenin accumulates (Recourt et al. 1989). Point mutation of NodD proteins extends *nod* gene expression to include flavonoids which are usually non-inducing (Burn et al. 1987; McIver et al. 1989). Together with additional research this has led to the suggestion that NodD controls rhizobial responses to flavonoids in a species-specific fashion (Horvath et al. 1987; Spaink et al. 1987; Zaat et al. 1989). However, the direct biochemical interaction of NodD and flavonoids has been difficult to prove, although more recent work has begun to demonstrate this interaction (Li et al. 2008; Peck et al. 2006). The work of Peck et al. (2006) has importantly shown that inducing and non-inducing flavonoids promote binding of *S. meliloti* NodD1 to the *nod* box, suggesting that competitive inhibition between inducing and non-inducing flavonoids may be important in regulating *nod* gene expression and thus nodulation efficiency. In vivo binding of *S. meliloti* NodD1 to the *nod* box upon luteolin treatment also requires the activity of the chaperonin GroEL (Ogawa and Long 1995; Yeh et al. 2002).

Flavonoid induction of NodD via the *nod* box has been well characterised, and 14 of the 16 genes required for Nod factor biosynthesis are regulated in this manner in *Sinorhizobium* sp. strain NGR234 (Freiberg et al. 1997; Kobayashi et al. 2004). The promoters of many other *Rhizobium* genes also contain *nod* boxes, as demonstrated in NGR234 which responds by increasing transcription of 147 open reading frames upon daidzein treatment (Perret et al. 1999). Indeed, flavonoid treatment can also act to repress gene expression (Firmin et al. 1986), for example coumestrol and medicarpin, flavonoids secreted by *M. sativa* roots, repress *nodC* expression in *S. meliloti* (Zuanazzi et al. 1998). Different flavonoids therefore play different roles to positively and negatively regulate *nod* gene expression in *Rhizobium* species.

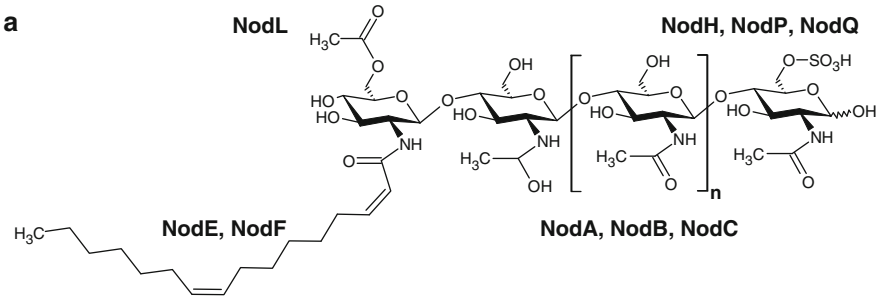
Transient increases in intracellular calcium in *R. leguminosarum* bv. *viciae* have recently been detected upon treatment with flavonoid inducers (Moscatiello et al. 2010). These calcium transients were NodD independent, suggesting that an additional flavonoid-perception mechanism remains to be characterised in *R. leguminosarum* bv. *viciae*. Flavonoid non-inducers did not activate the calcium response in *R. leguminosarum* bv. *viciae* (Moscatiello et al. 2010), therefore this alternative flavonoid-perception mechanism must be specifically activated by only flavonoid inducers. It will therefore be interesting to know the exact interplay between different flavonoids, NodD and calcium in the role of *nod* gene induction.

Silencing of enzymes involved in the biosynthesis of flavonoids has confirmed the importance of these secondary metabolites in establishing symbioses with rhizobia. *Medicago truncatula* plants silenced for chalcone synthase, chalcone reductase and flavone synthase II expression show decreased or no nodulation with *S. meliloti* (Wasson et al. 2006; Zhang et al. 2009). Similar silencing experiments in *Glycine max* also show decreased nodulation (Subramanian et al. 2006). These results are consistent with the importance of flavonoids in activating Nod factor biosynthesis, although it has also been suggested that auxin transport in host roots may be regulated by flavonoids and this has been proposed to be involved in nodule organogenesis (Subramanian et al. 2007). However, the relative importance of flavonoids activating Nod factor biosynthesis versus regulating auxin for nodule organogenesis remains to be resolved.

Non-flavonoid diffusible signals are also produced by legumes and perceived by rhizobia, although their role appears to be relatively minor due to the higher concentrations required for biological activity (Brencic and Winans 2005; Cooper 2007). The first non-flavonoid compounds to be identified were the betaines trigonelline and stachydrine from *M. sativa* which activated *nod* gene expression in *S. meliloti* (Phillips et al. 1992). Jasmonates also stimulate *nod* gene expression in *R. leguminosarum* (Rosas et al. 1998) and *Bradyrhizobium japonicum* (Mabood et al. 2006), whilst *B. japonicum nod* gene induction has also been described with xanthones (Yuen et al. 1995). Interestingly, simple phenolics from wheat, such as vanillin and isovanillin, are also able to act as *nod* gene inducers in *Sinorhizobium* sp. strain NGR234 (Le Strange et al. 1990).

2.2 *Nod Factors*

Upon perception of flavonoids, rhizobial NodD proteins induce *nod* gene transcription. Some of these *nod* gene products are enzymes involved in the production of a suite of lipochitooligosaccharides (LCOs) called Nod factors. The first Nod factor structure to be presented was that of *S. meliloti* (Fig. 2a; Lerouge et al. 1990). Nod factors have a generalised structure consisting of a chitin backbone of usually three to five *β-1,4*-linked *N*-acetylglucosamine residues to which additional decorations and substituents are added, including an acyl (fatty acid) chain at the non-reducing terminus. These decorations vary between *Rhizobium* strains and may include the addition of acetyl, methyl, sulfate and sugar moieties (Fig. 2b). A single species of rhizobia may produce several different Nod factors; for example, *Rhizobium tropici* CIAT899 produces 52 different LCOs at acidic pH and 29 LCOs at neutral pH, yet only 15 structures are common to both growth conditions (Morón et al. 2005).



Acyl	R_1	R_2	R_3	R_4	R_5	R_6	n
C16, C18, C20 (varying degrees of saturation; between 0 and 4 double bonds)	H, Me	H, OH, Cb	H, Ac, Cb	H, S, Ac, Fuc, MeFuc, AcFuc, AcMeFuc, SMeFuc	H, Ara	H, OH, Ac, Fuc	-1, 0, 1, 2, 3

Fig. 2 (**a**) Structure of *Sinorhizobium meliloti* Nod factor. The *N*-acetylglucosamine backbone is synthesised by NodA, NodB and NodC; the acyl chain group is determined by Nod E and NodF; *O*-sulfation is performed by NodH, NodP and NodQ; *O*-acetylation is performed by NodL. (**b**) Generalised structure of naturally occurring Nod factors. Table shows major decorations and variations in Nod factor structure based on species studied to date. *Ac* acetyl, *Ara* arabinosyl, *Cb* carbamoyl, *Fuc* fucosyl, *H* hydrogen, *Me* methyl, *OH* hydroxyl, *S* sulfate, *AcFuc* acetylated fucose, *MeFuc* methylfucose, *AcMeFuc* acetylated methylfucose, *SMeFuc* sulfated methylfucose. Figure adapted from Perret et al. (2000) and Wais et al. (2002)

Rhizobia also produce many other compounds (including type I and type III secreted proteins and surface polysaccharides) which have varying degrees of importance during nodulation. These will not be discussed here as Nod factors are the most important diffusible signalling molecules when considering the establishment of the legume-*Rhizobium* symbiosis. A recent detailed review of these additional signals is provided by Downie (2010).

2.2.1 Nod Factor Core (NodA, NodB, NodC)

The core structure of Nod factors is produced by the enzymes encoded for by the *Rhizobium* genes *nodABC*, which form an operon in many species. The first step of Nod factor biosynthesis involves the assembly of the chitin backbone through the activity of NodC, an *N*-acetylglucosaminyltransferase, which causes chain elongation at the non-reducing terminus (Kamst et al. 1997, 1999; Mergaert et al. 1995; Spaink et al. 1994). NodB, an *N*-deacetylase, then removes the *N*-acetyl moiety from the non-reducing terminus (John et al. 1993; Spaink et al. 1994), allowing for the subsequent addition of an acyl chain to the chitin oligomer via NodA, an *N*-acyltransferase (Fig. 2a; Atkinson et al. 1994; Debelle et al. 1996; Rohrig et al. 1994). NodA represents a unique biosynthetic enzyme since it allows the addition of an acyl chain to a polysaccharide without going through a nucleotide-activated intermediate.

The *nodABC* genes are absolutely essential for Nod factor synthesis and bacterial mutants in these genes are unable to initiate plant signalling (Wais et al. 2002) or nodulate their host (Fisher et al. 1985; Marvel et al. 1985). Cross-species complementation experiments have demonstrated that *nodABC* loci of a number of *Rhizobium* strains are able to complement mutants of a different strain and allow successful nodulation (Krishnan and Pueppke 1991; Marvel et al. 1985). Therefore, *nodABC* were previously referred to as the common nodulation genes. However, recent sequencing of two *Bradyrhizobium* species (BTAi1 and ORS278) revealed the absence of the *nodABC* genes from these genomes; despite this lack of *nodABC*, *Bradyrhizobium* species ORS285 efficiently formed fully functional nodules (Giraud et al. 2007). This exception to the rule therefore provides a unique example of Nod factor-independent establishment of a root nodule symbiosis.

NodA and NodC have also been implicated in determining host-range specificity: *S. meliloti* NodA is able to transfer unsaturated C16 fatty acids to the *N*-acetylglucosamine backbone whilst *R. tropici* NodA is unable to perform this reaction (Roche et al. 1996). Characterisation of NodC from different *Rhizobium* species has demonstrated that this protein controls the number of *N*-acetylglucosamine residues which condense together: *S. meliloti* NodC forms chitotetraoses whilst *Mesorhizobium loti* NodC forms chitopentaoses (Kamst et al. 1995, 1997). However, it is the decorations added to the core Nod factor structure which play a more important role in determining host-range specificity.

2.2.2 Nod Factor Decorations

Research into Nod factor structural requirements began through a series of cross-inoculation experiments whereby *Rhizobium* isolates from one legume were inoculated onto other legume species to determine whether nodulation was possible. Although the precise structures of Nod factors had not been determined, these experiments provided a wealth of data on host-range specificity. Now that many of these Nod factor structures have been determined it is clear that the decorations added to the core Nod factor structure (Fig. 2b) are important for determining host-range specificity. For example, expression of *nodABC* in *Escherichia coli* is alone sufficient to trigger root hair deformation in clover but not *M. sativa*; however, root hair deformation is observed in both species upon the additional expression of *nodH* (Banfalvi and Kondorosi 1989). Similar experiments have established roles for other host-specific *nod* genes in controlling host-range specificity through the decorations added to the core Nod factor structure (Lopez-Lara et al. 1996; Lorquin et al. 1997a, b; Mergaert et al. 1996; Spaink et al. 1991). Additionally, this role for different Nod factor structures in determining host-range specificity is particularly important when considering the broad host-range *Sinorhizobium* sp. strain NGR234 which is able to nodulate many host plants because it produces a wide variety of Nod factor structures (Price et al. 1992).

Acylation (NodE, NodF)

NodE and NodF determine which acyl chain(s) are added to the core Nod factor structure by NodA. NodF is an acyl carrier protein, whilst NodE is a β-ketoacyl synthase implicated in determining the degree of acyl chain saturation (Bloemberg et al. 1995a; Debelle and Sharma 1986; Geiger et al. 1991; Ritsema et al. 1997; van der Drift et al. 1996). In *R. leguminosarum* bv. *viciae,* NodE activity leads to the production of a Nod factor with a polyunsaturated C18:4 acyl chain. Inactivation of this gene instead results in the incorporation of vaccenic acid, an unsaturated C18:1 acyl chain (Spaink et al. 1991), and subsequently renders the strain unable to nodulate *Vicia sativa* (Canter Cremers et al. 1989). Deletion of *S. meliloti nodF* yields Nod factors with similar acyl chain compositions to those obtained from *nodE* deletion mutants, suggesting that the combined action of NodE and NodF is required for appropriate acyl chain addition (Demont et al. 1993). Indeed, exchanging the *nodEF* genes of *S. meliloti* with those of *R. leguminosarum* bv. *viciae* extends the production of Nod factors to include structures with polyunsaturated C18:2, C18:3 and C18:4 acyl chains (Demont et al. 1993). Methyl-branched acyl chains are added to the Nod factor of the arctic *Mesorhizobium* sp. strain N33 (*Oxytropis arctobia*) and this requires a fully functional *nodE* gene (Poinsot et al. 2001). Poinsot et al. (2001) speculate that incorporation of these unusual acyl chains is important for this species to tolerate extreme cold.

Some species of legumes utilise specific recognition of the acyl chain as a stringent measure of Nod factor during rhizobial infection (Ardourel et al. 1994; Walker and Downie 2000). However, this recognition occurs in combination with either the *O*-acetylation of Nod factor by NodL (Sect. 2.2.2.3; Ardourel et al. 1994), or the action of other Nod proteins (Walker and Downie 2000).

Glycosylation (NoeC, NodZ, NolK)

Two forms of glycosylation have been described as Nod factor decorations: arabinosylation and fucosylation. The importance of both modifications has been described for Nod factors from *Azorhizobium caulinodans*, the symbiont of *Sesbania rostrata* (Mergaert et al. 1997). Arabinosylation and fucosylation also appear to be required by other symbionts of *S. rostrata*, suggesting a possible responsibility of these glycosyl decorations for determining host-range specificity (Lorquin et al. 1997a).

D-arabinosylation on C3 of the reducing terminus of Nod factor is dependent on *noeC* and/or downstream genes in *A. caulinodans* (Mergaert et al. 1996). Presence of this *D*-arabinosyl group on Nod factors from *A. caulinodans* results in higher numbers of nodules on *S. rostrata* roots than Nod factors without this decoration (Fernandez-Lopez et al. 1998). However, other host species of *A. caulinodans* show a preference for fucosylated Nod factors, implying that arabinosylation is particularly important for nodulation of *S. rostrata* (Fernandez-Lopez et al. 1998).

L-fucosylation of *A. caulinodans* Nod factors on C6 of the reducing terminus depends on both *nodZ* and *nolK*. NolK is involved in the biosynthesis of GDP-fucose, which is a substrate for the fucosyltransferase NodZ (Mergaert et al. 1996). These authors also detected a NodZ-independent Nod factor fucosyltransferase activity, although this activity was not encoded for by any of the known *nod* genes.

NodZ also decorates the Nod factors of other *Rhizobium* species with fucosyl groups (Lopez-Lara et al. 1996; Quesada-Vincens et al. 1997; Quinto et al. 1997; Stacey et al. 1994). NodZ from *Sinorhizobium* sp. strain NGR234 preferentially fucosylates chitopentaoses over single *N*-acetylglucosamine residues or non-fucosylated Nod factors, implying that fucosylation occurs before acylation (Quesada-Vincens et al. 1997). Nodulation of *Macroptilium atropurpureum* by *B. japonicum* (Stacey et al. 1994) and *Pachyrhizus tuberosus* by *Sinorhizobium* sp. strain NGR234 (Quesada-Vincens et al. 1997) is blocked by mutation of *nodZ*. However, expression of *B. japonicum nodZ* in *R. leguminosarum* bv. *viciae* results in fucosylated Nod factor production and extends the host range of the *R. leguminosarum* strain (Lopez-Lara et al. 1996). It is also interesting to note that despite the absolute necessity of *B. japonicum* for NodZ in order to nodulate *M. atropurpureum*, *nodZ* expression is not controlled by NodD, a trait which is unique amongst the *nod* genes (Stacey et al. 1994).

An additional rare fucosylation site has been identified in *Mesorhizobium loti* strain NZP2213 where the fucosyl residue is found on a non-terminal *N*-acetylglucosamine residue of the Nod factor structure (Olsthoorn et al. 1998).

Acetylation (NodL, NodX, NolL)

An *O*-acetyl group can be added on C6 of either the reducing or non-reducing terminal *N*-acetylglucosamine residue of Nod factor through the action of NodX or NodL, respectively (Bloemberg et al. 1994). NodX from *R. leguminosarum* bv. *viciae* is able to only use chitopentaoses as a substrate for *O*-acetylation (Firmin et al. 1993) and the quantities of *O*-acetylated Nod factor produced by NodX is temperature-dependent (Olsthoorn et al. 2000). Mutation of *nodX* in *R. leguminosarum* bv. *viciae* strain TOM abolishes the ability of this strain to nodulate *Pisum sativum* cv. Afghanistan (Davis et al. 1988). This *nodX* mutant produces other LCOs identical to wild-type bacteria (Ovtsyna et al. 1999), suggesting that the specificity of the interaction between strain TOM and *P. sativum* cv. Afghanistan is due to *O*-acetylation. However, nodulation with this *nodX* mutant can be restored by expression of *nodZ* from *B. japonicum* (Sect. 2.2.2.2; Ovtsyna et al. 1998), implying that *O*-acetylation alone cannot be the only mechanism for determining specificity in this interaction.

The NodL *O*-acetylation reaction is dependent upon a non-reducing terminally de-*N*-acetylated chitin oligosaccharide substrate (i.e. the product of NodB and NodC activity; Bloemberg et al. 1995b). The stringency for NodL-mediated *O*-acetylation appears to be low since fully functional nodulation of *M. sativa* by the *S. meliloti nodL* mutant is possible, although significantly decreased infection thread formation and a delay in nodulation was noted (Ardourel et al. 1994).

In addition to acetylation by NodX or NodL, acetyl groups can be added onto fucose decorations via NolL. NolL from *Sinorhizobium* sp. strain NGR234 leads to Nod factor structures with 3-*O*- or 4-*O*-acetylation on fucose (Berck et al. 1999), whilst NolL from *Rhizobium etli* yields Nod factors with only 4-*O*-acetylation on fucose (Corvera et al. 1999). NolL is not essential for nodulation of *Phaseolus vulgaris* by *R. etli*, although nodulation of the *nolL* mutant was less efficient than the wild-type strain on some *P. vulgaris* cultivars (Corvera et al. 1999). Heterologous expression of *nodZ* or *nodZ* and *nolL* has demonstrated that NolL is necessary for efficient nodulation of *Lotus japonicus* by *R. leguminosarum* bv. *viciae* (Pacios Bras et al. 2000). Different *Lotus* species also have different requirements for NolL-mediated acetylation on fucose: the *M. loti nolL* mutant is unable to form infected nodule primordia on *L. filicaulis* and *L. corniculatus* yet can successfully nodulate *L. japonicus* (Rodpothong et al. 2009). This apparent discrepancy for the requirement of NolL for successful nodulation of *L. japonicus* was explained by Rodpothong et al. (2009) as being due to other differences, notably the acyl chain structure, between the Nod factors of *M. loti* (the true symbiont of *L. japonicus*) and *R. leguminosarum* bv. *viciae* expressing *nodZ* and *nolL*.

A rare acetylation site has been determined in the *M. loti* strain N33 where *6-O*-acetylation occurs on the residue proximal to the non-reducing *N*-acetylglucosamine, although the gene encoding the enzyme responsible for this modification has not been identified (Poinsot et al. 2001).

Methylation (NodS, NoeI)

Two forms of methylation can occur on Nod factors: *N*-methylation on the non-reducing terminus controlled by NodS (Geelen et al. 1993; Jabbouri et al. 1995) or *2-O*-methylation on fucose mediated by NoeI (Jabbouri et al. 1998). NodS is an *N*-methyltransferase and in *A. caulinodans* or *Sinorhizobium* sp. strain NGR234 methylates end-deacetylated chitooligosaccharides using an *S*-adenosyl-*L*-methionine-binding protein as a methyl donor (Geelen et al. 1995). Indeed, the structure of NodS from *B. japonicum* has recently been solved, representing the first crystal structure of an *S*-adenosyl-*L*-methionine-dependent methyltransferase (Cakici et al. 2010). The *R. etli nodS* mutant is less able to induce root hair curling and actin cytoskeleton rearrangements in *P. vulgaris* than wild-type *R. etli*, suggesting that *N*-methylation is key in regulating these Nod factor-dependent responses (Cardenas et al. 2003). *N*-methylation by NodS biosynthetically precedes any *O*-acetylation reactions by NodL (Lopez-Lara et al. 2001).

2-O-methylation of fucose by NoeI is common in *Sinorhizobium* sp. strain NGR234 and *S. fredii* strain USDA257 (Jabbouri et al. 1998). Mutation of this gene leads to production of LCOs which are non-methylation on fucose, although as this appears to have no effect on nodulation (Jabbouri et al. 1998) this Nod factor decoration is of lesser significance.

Carbamoylation (NodU, NolO)

Carbamoylation on the non-reducing terminal *N*-acetylglucosamine residue is controlled by the carbamoyltransferases NodU and NolO. Expression of *Sinorhizobium* sp. strain NGR234 *nodU* in *S. fredii* strain USDA257 (which does not produce carbamoylated Nod factors) allows *6-O*-carbamoylated Nod factors production (Jabbouri et al. 1995). Likewise, expression of *Sinorhizobium* sp. strain NGR234 *nolO* in *S. fredii* strain USDA257 has confirmed the role of NolO in controlling *3-O*- and *4-O*-carbamoylation at the non-reducing terminus (Jabbouri et al. 1998). The host range of *S. fredii* expressing *nolO* is increased to include non-host species (Jabbouri et al. 1998). However, the nodulation phenotype of the *S. fredii nolO* mutant was not different from the wild-type strain, although the mutant showed decreased competitiveness to nodulate *G. max* (Madinabeitia et al. 2002). Interestingly, Jabbouri et al. (1998) suggest the existence of a third (as yet uncharacterised) carbamoyltransferase in *Sinorhizobium* sp. strain NGR234 since mutation of *nodU* and *nolO* failed to result in Nod factors entirely devoid of carbamoylation.

Sulfation (NodH, NodP, NodQ, NoeE)

Addition of an *O*-sulfate group is common to many Nod factors and this reaction is performed by the sulfotransferases NodH (Del Papa et al. 2007; Ehrhardt et al. 1995; Laeremans et al. 1996; Lerouge et al. 1990; Roche et al. 1991; Schultze et al.

1995) and NoeE (Hanin et al. 1997). NodH activity results in sulfation on C6 of the reducing terminus, while NoeE only gives sulfation on fucose residues attached to C6 of the reducing terminus (Quesada-Vincens et al. 1998). NodP and NodQ are also essential for Nod factor sulfation and act as sulfur activators by synthesising the sulfur donor 3′-phosphoadenosine 5′-phosphosulfate (Schwedock and Long 1990; Schwedock et al. 1994).

The *R. tropici* strain CFN299 *nodP* mutant shows decreased nodulation on *P. vulgaris* cv. Negro Xamapa, while *nodH* and *nodP* mutants acquire an increased capacity to nodulate the two other cultivars (Laeremans et al. 1996). Likewise, the *nodH* mutant of *R. tropici* shows decreased nodulation in comparison to wild-type when nodulating *Leucaena leucocephala* (Folch-Mallol et al. 1996). *R. fredii* expressing *noeE* produced sulfated LCOs and therefore acquired the ability to nodulate *Calopogonium caeruleum*, whilst mutation of *noeE* from *Sinorhizobium* sp. strain NGR234 abolished the production of sulfated LCOs and prevented nodulation of *P. tuberosus* (Hanin et al. 1997). Importantly, *S. meliloti* Nod factor sulfation is essential for root hair deformation and nodulation of *M. sativa* (Roche et al. 1991). These findings all support a role for sulfation as a major determinant of symbiont specificity for their host plant species, yet in other interactions the stringency for sulfation appears to be low. Mutation of the *nodHPQ* genes of *Rhizobium* sp. strain N33 appears to have no effect on nodulation of two host species tested (Cloutler et al. 1996). Remarkably, a recent report suggests that the *nodH* mutant of *Sinorhizobium* sp. strain BR816 shows increased nitrogen fixation relative to the wild-type strain despite there being no other nodulation phenotype (Remans et al. 2007). The authors attribute this interesting result to the availability of activated sulfate inside nodules which they argue is likely to be greater with the *nodH* mutant.

2.2.3 Nod Factor Secretion

NodI and NodJ act as an ATP-binding cassette (ABC) transporter (Higgins et al. 1986) and are involved in Nod factor secretion. Secretion of Nod factor is impaired in *nodI* and/or *nodJ* rhizobia mutants (Cardenas et al. 1996; Fernandez-Lopez et al. 1996; Spaink et al. 1995), whilst *E. coli* engineered for the biosynthesis of Nod factors only secreted these LCOs in the presence of NodI and NodJ (Fernandez-Lopez et al. 1996). Interestingly, the *nodIJ* mutant of *R. etli* is able to nodulate *P. vulgaris*, although the mutant shows a delayed and decreased nodulation phenotype in comparison to the wild-type. This non-essential role of NodI and NodJ therefore suggests a possible additional component involved in rhizobial secretion of Nod factors which has yet to be characterised.

2.3 Plant Responses to Nod Factor

Nod factors are perceived in epidermal and root hair cells since fluorescent Nod factors added to plant roots accumulate in the walls of these cells (Goedhart et al. 2000). Host plant cells are able to perceive Nod factor concentrations as low as 10^{-12} M (Oldroyd and Downie 2004), suggesting that the receptor able to perceive Nod factors is highly sensitive. In *L. japonicus,* two Nod factor receptors have been identified: NFR1 and NFR5 (Madsen et al. 2003; Radutoiu et al. 2003). The *M. truncatula* gene equivalent to *NFR5* is *NFP* (Nod factor perception), whilst *LYK3* is orthologous to *NFR1* (Amor et al. 2003; Smit et al. 2007). Interestingly, LYK3 has been described as an entry receptor which controls rhizobial infection in a manner dependent upon Nod factor structure (Smit et al. 2007), therefore suggesting an additional element for Nod factor structural specificity in establishing the legume-*Rhizobium* symbiosis. These Nod factor receptors are receptor-like kinases and contain LysM domains which are involved in binding *N*-acetylglucosamine, making them likely Nod factor receptors. Importantly, *M. truncatula* transformed with *NFR1* and/or *NFR5* was able to form nodules with a rhizobial species usually specific to *L. japonicus* (Radutoiu et al. 2007). This directly implicates NFR1 and NFR5 as Nod factor receptors whilst also demonstrating the importance of these receptors and the structure of Nod factors themselves for determining symbiont specificity. However, binding assays between Nod factors and their potential targets have yet to provide formal evidence for a direct physical interaction between ligand and receptor.

Nod factors trigger a range of molecular responses in legumes (reviewed by D'Haeze and Holsters 2002; Oldroyd et al. 2001a). These responses include rapid pH changes (Felle et al. 1996, 2000), root hair deformation (Roche et al. 1991; Spaink et al. 1991), lateral root formation (Olah et al. 2005), reactive oxygen species production (Cardenas et al. 2008; Cardenas and Quinto 2008), induction of calcium flux (Ehrhardt et al. 1992), induction of calcium spiking (Ehrhardt et al. 1996), and gene expression changes (Mitra et al. 2004). Nod factors are required for infection thread development but are insufficient to activate this response alone (Dazzo et al. 1991), although the formation of pre-infection thread structures has been described (van Brussel et al. 1992). At sufficiently high concentrations Nod factors can also induce cortical cell division and the formation of nodule primordia (Truchet et al. 1991). The diversity of these responses only goes to demonstrate the critical importance of Nod factors as signalling molecules in the early stages of nodulation.

The sensitivity of the responses triggered by Nod factor can vary by several orders of magnitude; for example, the two Ca^{2+} signatures (flux and spiking) can be separated, such that high concentrations ($>10^{-9}$ M) of Nod factor induce flux followed by spiking, while low Nod factor concentrations ($<10^{-10}$ M) induce only calcium spiking (Shaw and Long 2003). *Pisum sativum* plants treated with chitin oligomers of four or five residues also show calcium spiking, but not calcium flux (Walker et al. 2000). These observations suggest that the activation of calcium

spiking has a lower stringency for Nod factor structure and concentration than the induction of calcium flux. The structural requirements of *S. meliloti* Nod factors to trigger calcium spiking have been analysed and effects due to missing decorations, such as *O*-acetylation, *N*-acylation or *O*-sulfation, can be overcome by treating with high enough concentrations of Nod factor (Oldroyd et al. 2001b; Wais et al. 2002). This work has formally shown that Nod factor decorations, in addition to determining host-range specificity, play a role in determining the potency of the Nod factor signal to the plant and that concentration of LCOs must therefore be considered when determining biological activity.

Calcium spiking, the rapid oscillation of calcium concentration in the nucleus and peri-nuclear region of root hair cells (Ehrhardt et al. 1996), is central to the signalling pathway activated by Nod factor (Oldroyd and Downie 2004). Components of this common symbiosis signalling pathway are required for both nodulation and mycorrhization in legumes; mutation in any of these genes blocks the formation of either symbiosis.

3 Diffusible Signals During Mycorrhization

The molecular dialogue between AM fungi and host plant roots has been less well characterised than that of legumes and rhizobia, partly because the fungus is an obligate biotroph which makes it less amenable to study. An additional problem for the study of AM fungi is the asynchronous nature of the infection process. Despite these limitations, research in this area has begun to provide some interesting parallels between signalling during nodulation and mycorrhization (reviewed by Bonfante and Genre 2010; Harrison 2005; Parniske 2008). Diffusible signals again play a key role in the establishment of mycorrhizal interactions. Strigolactones are released from plant roots and these promote AM fungal spores to germinate. Germinated spores produce a diffusible signal, a so-called "Myc" factor, which triggers signalling in the plant (mediated by the symbiosis signalling pathway in both legumes and non-legumes; Gutjahr et al. 2008). The chemical nature of "Myc" factor has proved elusive, although recent work has characterised LCOs derived from AM fungi which act in an analogous fashion to Nod factor.

3.1 Strigolactones

Strigolactones play an important role in establishing mycorrhizal symbioses, serving as germination and hyphal branching cues for dormant AM fungal spores. Nothing is known about strigolactone perception by the fungus or the requirement for different chemical structures of strigolactones. Other diffusible signals, including flavonoids, have also been implicated in triggering spore germination.

3.1.1 Structure and Synthesis

The first strigolactone isolated from root exudates was strigol (Cook et al. 1966). Numerous subsequent experiments have demonstrated the presence of strigolactones in root exudates from different species, including both monocotyledonous (Awad et al. 2006) and dicotyledonous plants (Yoneyama et al. 2008). It was not until 2003 though that strigolactones were formally proved to be derived from roots, as demonstrated through aseptic plant culture experiments (Yasuda et al. 2003). Many chemical structures of naturally occurring strigolactones have now been proposed (Yoneyama et al. 2009); these vary primarily in the position and number of hydroxyl, methyl and acetyl groups present on the core ring structure. Interestingly, *Arabidopsis thaliana*, a non-mycorrhizal species, produces low concentrations of strigolactones relative to other plant species which can form symbioses with AM fungi (Westwood 2000).

Relatively little is known about the biosynthesis of strigolactones, which were originally considered to be a group of sesquiterpene lactones. However, the tricyclic ring structure of strigolactones is now known to be derived from carotenoid biosynthesis (Fig. 3; Jamil et al. 2010; Lopez-Raez et al. 2008; Matusova et al. 2005). Two proteins characterised in *A. thaliana* as carotenoid cleavage dioxygenase enzymes have been implicated specifically in the biosynthesis of strigolactones: CCD7 (Booker et al. 2004) and CCD8 (Sorefan et al. 2003). *CCD7* and *CCD8* were originally studied for their mutant phenotypes in shoot branching and a role for branching inhibition by strigolactones is now established (Gomez-Roldan et al. 2008; Umehara et al. 2008). Mutation or silencing which causes decreased *CCD7* expression in tomato plants gives rise to strigolactone-impaired lines which show decreased colonisation with AM fungi, thus demonstrating the importance of this enzyme for the synthesis of strigolactones and the importance of strigolactones for mycorrhization (Koltai et al. 2010; Vogel et al. 2010).

3.1.2 Biological Activity

Strigolactones have been well characterised for their role in the interaction between plants and weeds of the genus *Striga*, from where these molecules derive their name. During this parasitic interaction, strigolactones released by the host plant promote germination of *Striga* species (reviewed by Bouwmeester et al. 2003). However, the role of strigolactones during symbiotic interactions with AM fungi was not determined until 2005 (Akiyama et al. 2005).

The amount of strigolactone secretion by roots is thought to be very low, but these molecules are highly potent and are able to induce fungal hyphal branching at picogram to nanogram quantities (Akiyama and Hayashi 2006; Bucher et al. 2009). For this reason, synthetic strigolactones have also been used in the study of strigolactones and AM fungi. Fungal responses after the application of synthetic

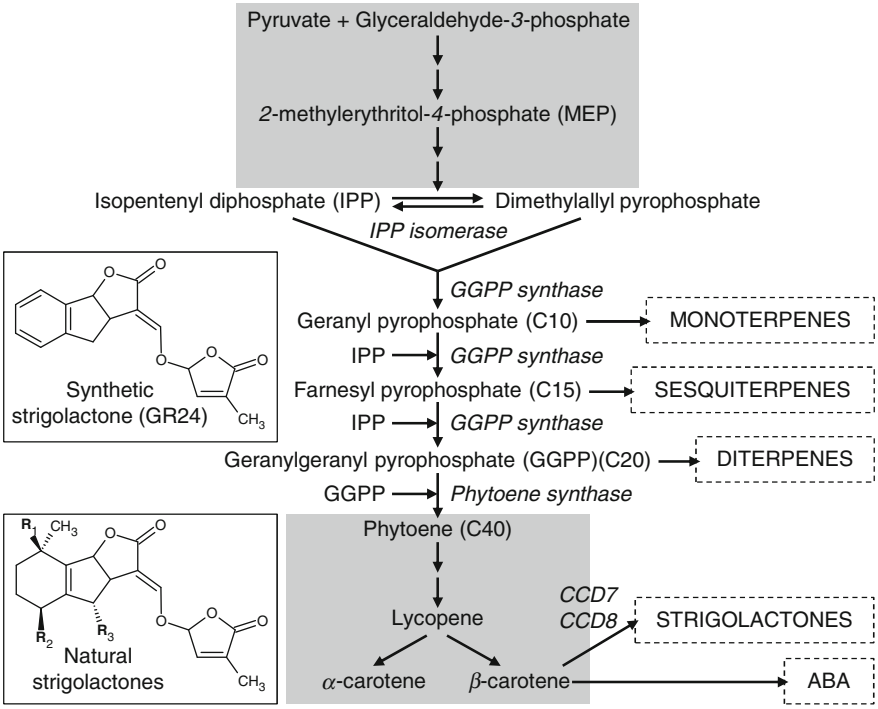


Fig. 3 Biosynthesis of strigolactones. Partial diagram of the carotenoid biosynthesis pathway. Enzymes are depicted in italics; the *2*-methylerythritol-*4*-phosphate (MEP) pathway and the first committed steps of carotenoid biosynthesis are denoted by upper and lower shaded boxes, respectively; compounds in dashed boxes represent major side branches of the pathway. *ABA* abscisic acid, *CCD7*/*8* carotenoid cleavage dioxygenase, *GGPP* geranylgeranyl pyrophosphate, *IPP* isopentenyl diphosphate. Insets show chemical structures of example synthetic and naturally occurring strigolactones: strigol: $R_1 = CH_3, R_2 = OH, R_3 = H$; strigyl acetate: $R_1 = CH_3, R_2 = OAc, R_3 = H$; sorgolactone: $R_1 = H, R_2 = H, R_3 = H$. Figure adapted from Akiyama and Hayashi (2006) and Matusova et al. (2005)

strigolactones, such as GR24, are comparable to treatment with naturally occurring strigolactones (Akiyama et al. 2005). Strigolactones (or "branching factors" as they were originally named before their identification) promote spore germination and branching in *Gigaspora* spp. (Akiyama et al. 2005; Buee et al. 2000; Nagahashi and Douds 1999). In addition, strigolactone treatment promotes a number of other fungal pre-symbiotic responses, including the induction of mitosis (Buee et al. 2000), increased expression of mitochondrial-related genes (Tamasloukht et al. 2003), increased density of mitochondria (Besserer et al. 2009), and thus increased respiratory activity (Tamasloukht et al. 2003).

Mycorrhization is impaired in the strigolactone-deficient *ccd8* mutant of *P. sativum*, although this phenotype can be partially recovered by exogenous application of GR24 (Gomez-Roldan et al. 2008). As full complementation is not achieved with exogenous treatment of strigolactone it is possible that directionality

of the diffusible signal via a concentration gradient is important in order to encourage germinating AM spores to grow towards host roots. This existence and importance of a natural concentration gradient is especially likely when considering that strigolactones are readily hydrolysed in the soil (Akiyama and Hayashi 2006). Evidence for chemotaxis responses of AM fungi to root diffusible signals supports this hypothesis (Sbrana and Giovannetti 2005).

The involvement of flavonoids as diffusible signals during the establishment of interactions with AM fungi remains unclear (as discussed by Larose et al. 2002; Vierheilig et al. 1998). For example, the flavonoid medicarpin accumulates in *M. sativa* roots and strongly inhibits *Glomus intraradices* hyphal growth (Guenoune et al. 2001), whilst a flavonoid from melon roots enhances mycorrhization in this species (Akiyama et al. 2002). Likewise, the flavonoid quercetin stimulates AM fungal spore growth and branching (Becard et al. 1992; Tsai and Phillips 1991). Other studies have suggested that flavonoids are not absolutely essential for hyphal growth (Becard et al. 1995). The most likely conclusion is that AM fungal responses to flavonoids are compound and genus specific (Scervino et al. 2005a, b), therefore making it difficult to assign a definitive role for these diffusible signals during the establishment of symbioses with AM fungi. Contrasting this with nodulation, recent evidence suggests that nodulation of *M. sativa* by *S. meliloti* is increased by strigolactone treatment (Soto et al. 2010); it will therefore be interesting to know whether the interplay between these diffusible signals is important for the establishment of both symbioses.

3.2 "Myc" Factors

A diffusible signal originating from the fungus, the so-called "Myc" factor, has long been hypothesised, but until recently had not been characterised. It has been shown that a diffusible factor released from germinating AM fungi is able to induce expression of *ENOD11*, a symbiosis-specific gene in *M. truncatula* (Kosuta et al. 2003). Olah et al. (2005) used a membrane separating AM fungi from plants to demonstrate that this diffusible fungal factor identified by Kosuta et al. (2003) activates root branching in *M. truncatula* (a response also observed with Nod factor). AM fungi are able to induce calcium spiking in *M. truncatula* root hair cells associated with highly branched fungal hyphae and this occurs prior to physical contact between the fungus and the root (Kosuta et al. 2008). Calcium spiking has also been detected upon contact of fungal hyphopodia with non-trichoblastic root cells of *M. truncatula* and *Daucus carota*, and also upon treatment of *M. truncatula* roots with a concentrated extract from germinating fungal spores (Chabaud et al. 2011). This Ca^{2+} spiking in *M. truncatula* is dependent on components of the symbiosis signalling pathway but is *NFP*-(in)dependent, therefore implying different plant machineries for the recognition of "Myc" and Nod factors (Chabaud et al. 2011; Kosuta et al. 2008). Calcium transients have also been detected in *G. max* cell cultures exposed to germinating spores from *Glomus* species

(Navazio et al. 2007), although this signal was also released by non-germinating spores so perhaps represents a triggering of defence responses.

3.2.1 LCOs as a "Myc" Factor

An exciting recent development has proposed the structures of two LCOs produced by the AM fungus *G. intraradices* (Fig. 4; Maillet et al. 2011). One of these LCOs contains an unsaturated C18:1 acyl chain and is non-sulfated on the reducing terminus (Fig. 4a), whilst the other LCO has a saturated C16 acyl chain and is *O*-sulfated at the reducing terminus (Fig. 4b). These LCOs were characterised for their ability to induce *pENOD11::GUS* expression and root hair deformation in *M. truncatula*. Application of these Myc LCOs to *M. truncatula*, *Tagetes patula* or *D. carota* resulted in increased mycorrhizal colonisation. Increased lateral root formation in *M. truncatula* was also observed upon Myc LCO treatment and importantly this was *NFP*-dependent (Maillet et al. 2011). It has been previously

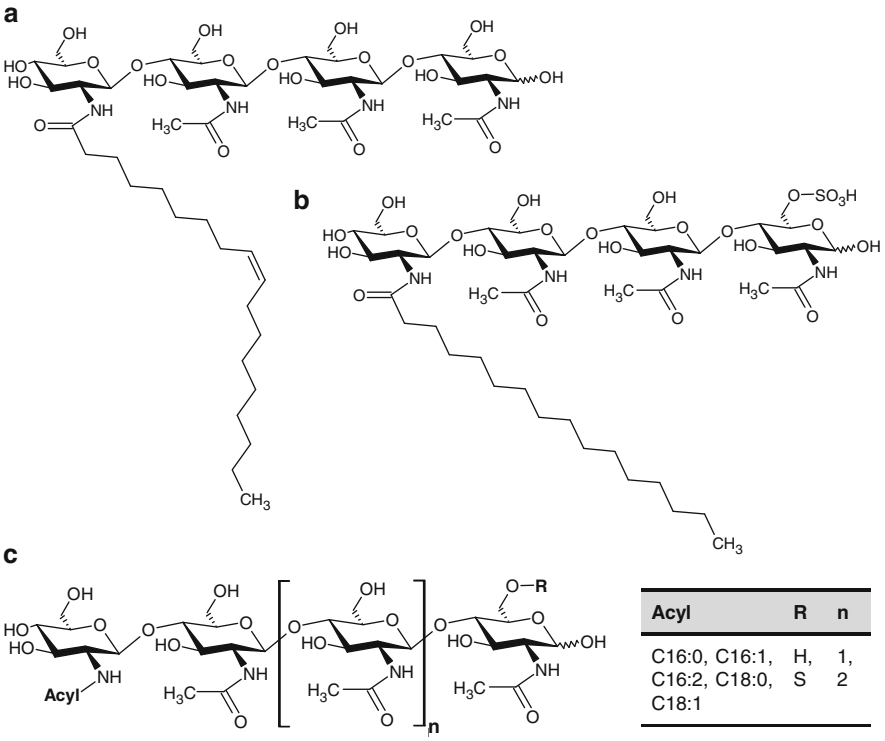


Fig. 4 (**a**) Structure of *Glomus intraradices* non-sulfated lipochitooligosaccharide (LCO). (**b**) Structure of *G. intraradices* sulfated LCO. (**c**) Generalised structure of naturally occurring *G. intraradices* LCOs. Table shows decorations and variations in Myc LCOs. Abbreviations as in Fig. 2. Figure adapted from Maillet et al. (2011)

shown that *NFP* is not required for mycorrhizal associations (Amor et al. 2003); therefore the apparent discrepancy for the requirement of *NFP* for inducing different mycorrhizal responses in *M. truncatula* could be due to the existence of other as yet uncharacterised diffusible signals derived from AM fungi. The *NFP*-independent diffusible fungal signals described by Olah et al. (2005) and Chabaud et al. (2011) may therefore represent different novel classes of diffusible signalling molecules (i.e. non-LCOs) involved in establishing the mycorrhizal symbioses. The apparent dual role of NFP in signalling during mycorrhization and nodulation could also be explained by the existence of a receptor complex consisting of NFP and other currently uncharacterised receptors. This is supported by the dual role of *Parasponia* NFP, which is involved in both nodulation and mycorrhization (Op den Camp et al. 2011). Alternatively, if early mycorrhizal signalling occurs in a directly analogous fashion to that of Nod factor, the "Myc" factor which causes *NFP*-independent root branching (Olah et al. 2005) and calcium spiking (Chabaud et al. 2011; Kosuta et al. 2008) would require an additional separate receptor. However, such a "Myc" factor receptor has yet to be identified.

Given the diversity of Nod factor structures and the fact that at least 80% of land plants are able to engage in symbiotic interactions with mycorrhiza, it is almost certain that other Myc LCOs exist in nature and have yet to be isolated and characterised. It is also tempting to speculate that *G. intraradices* and other AM fungi produce a broad spectrum of LCOs in order to colonise a wide range of host species, as with the broad host-range *Sinorhizobium* sp. strain NGR234.

4 Conclusions and Perspectives

The importance of diverse diffusible signals during the establishment of nodulation and mycorrhization is clear. Nod factor structural diversity has been implicated as a key determinate of host-range specificity, as discussed here and reviewed extensively by D'Haeze and Holsters (2002) and Perret et al. (2000). However, this structural diversity alone does not determine host-range specificity in all legume-*Rhizobium* symbioses; for example, *R. etli* and *M. loti* produce identical Nod factors yet nodulate different plant species (Cardenas et al. 1995). The recent identification of LCOs produced by the AM fungus *G. intraradices* will no doubt result in detailed research into the structural importance of these molecules during mycorrhizal interactions. Developments in signalling by nodulation- and mycorrhization-specific LCOs will also prove exciting, particularly in addressing the question of specificity in symbiosis signalling.

When considering the signals released from symbionts it is important to bear in mind that mycorrhization evolved ~400 million years before nodulation (Kistner and Parniske 2002). Chitin, the major component of fungal cell walls, therefore becomes a key molecule: chitin fragments released from cell walls of symbiotic or pathogenic fungi would act as a trigger of plant defence responses. In order to differentiate themselves as symbiotic (and also to protect against hydrolysis by

plant-released chitinases) it would be essential that AM fungi modify these structures with decorations. For rhizobia to adopt fungal genes responsible for Myc LCO production and thereby gain the ability to synthesise LCOs becomes a tangible explanation for the existence of Nod factors. It will therefore be interesting whether the genomes of AM fungi contain homologues to any rhizobial *nod* genes. The question then turns to pathogens and why these too have not evolved diffusible signals such as LCOs in order to hijack symbiotic signalling. Since plants regulate their symbioses by monitoring symbiotic efficiency (Schumpp and Deakin 2010) it is plausible that pathogenic microorganisms which do abuse this symbiotic molecular dialogue and gain entry to the root are detected and killed by the host species. Indeed, the combined action of diffusible LCOs and many additional non-diffusible signals is essential in order to discriminate between pathogenic and symbiotic microorganisms (Hamel and Beaudoin 2010).

In conclusion, diffusible signals from both host plant and symbiont are essential for the establishment of nodulation and mycorrhization. Work since the original characterisation of Nod factors has irrefutably demonstrated the importance of these rhizobial-derived LCOs during the legume-*Rhizobium* symbiosis. Future research into mycorrhizal-derived LCOs will no doubt identify novel LCO structures produced by AM fungi and hopefully begin to shed light on the mechanisms of discriminating specificity during symbiosis signalling.

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Infection of *Lotus japonicus* Roots by *Mesorhizobium loti*

Katharina Markmann, Simona Radutoiu, and Jens Stougaard

Abstract Like the two important crop legumes soybean and common bean, the model legume *Lotus japonicus* develops determinate root nodules. *L. japonicus* is normally infected through root hair infection threads in a process closely synchronised with the progressing primordial cell divisions and organ development. Recent studies of symbiotic mutants have however led to a remarkable and unexpected discovery of two alternative intercellular infection modes, crack entry and infection thread independent single cell infection, in *L. japonicus*. These results provide genetic support for the origin of rhizobial infection of legumes through direct intercellular epidermal invasion and indicate that this ancient infection process in subsequent evolutionary steps was surpassed by the Nod-factor dependent crack entry and root hair infection thread invasions observed in most extant legumes.

1 Introduction

Soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) are the two most important crop legumes for the world's production of food and feed. Like the model legume *Lotus japonicus*, both soybean and bean form determinate root nodules following inoculation with their respective rhizobial microsymbionts. A determinate nodule is characterised by a transient meristematic activity and cessation of infection thread growth after the majority of cells in the central zone of the primordia have been infected. The formation of a determinate root nodule is therefore a sequential process where clearly defined developmental stages replace each other and can thus be separated in time. In contrast to determinate nodules,

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which tend to be round at maturity, indeterminate nodules of for example pea, clover and a second model legume *Medicago truncatula* retain meristematic activity near the nodule tip and therefore develop into elongate structures. Underlying the tip meristem is the infection zone, where newly produced nodule cells are infected. Following the early stages founding the nodule primordium, an individual indeterminate root nodule will therefore represent spatially – but not temporally – separated stages.

Legume root nodule formation results from two tightly coordinated processes running in parallel. Development of nodule primordia from dedifferentiated root cortical cells initiates a meristem, which will later form the nodule. Simultaneously, a bacterial invasion process targets the primordia. In soybean and bean the outer cortical cells initiate primordia formation. In *L. japonicus* initial cell divisions were detected in cells of both the outer and middle cortex (Szczyglowski et al. 1998; van Spronsen et al. 2001). Cytoplasmic bridges preparing cells for infection thread passage were observed associated with primordia in *L. japonicus*, but not in bean (van Spronsen et al. 2001). Like soybean and bean, *L. japonicus* is normally infected through root hair infection threads, although capacities for crack entry and intercellular invasion were recently uncovered in studies of mutants (Karas et al. 2005; Groth et al. 2010; Madsen et al. 2010). The bacterial infection process starts with rhizobial attachment to plant root hair tips. Subsequent physiological and morphological responses of root hairs result in their curling, and bacteria are entrapped in an infection pocket at the curls' centre (Szczyglowski et al. 1998; Oldroyd and Downie 2004; Miwa et al. 2006). Starting from the pocket, inwards growing infection threads form and are colonised by rhizobia. These infection threads act as rhizobial conduits from which the *Mesorhizobium loti* symbiont ultimately will be released and endocytosed into nodule primordia cells. This infection mode targets pre-developed cells of the emerging nodule, and uninfected, so-called interstitial cells, are retained in between the infected cells of the central zone (Imaizumi-Anraku et al. 1997). As a result, infected cells constitute only a subset of cells in the central zone of determinate *L. japonicus*, bean and soybean nodules. In the infected cells rhizobia differentiate into bacteroids that are surrounded by a peribacteriod membrane derived from the infection thread membrane during endosymbiotic uptake. Together, bacteroids and membrane are referred to as symbiosomes, organelle-like structures where atmospheric dinitrogen is reduced to ammonium. Access to this reduced form of nitrogen provides the host with an ecological advantage over other plants under conditions where soil nitrogen resources are limiting. The *M. loti* bacteroids contained in *L. japonicus* nodules are not terminally differentiated and maintain their normal bacterial size, genome content and ability to divide (Van de Velde et al. 2010). This is in contrast to the indeterminate nodules of *M. truncatula*, where irreversible differentiation of bacteriods is thought to be promoted by an abundance of nodule-specific cysteine-rich anti microbial peptides (Van de Velde et al. 2010). *L. japonicus* nodules are devoid of these anti microbial peptides, which could be important in maintaining the continuous infection process in the indeterminate nodules (Van de Velde et al. 2010).

2 Recognition Precedes Infection

Several bacterial species belonging to both the α- and the ß-proteobacteria (Moulin et al. 2001; Broughton 2003) can induce root nodules on different legume species. The relationship between the approximately 18,000 legume species and their bacterial microsymbionts is nevertheless selective. *L. japonicus* can for example develop fully functional nitrogen fixing nodules with *M. loti* and ineffective or partially effective nodules with NGR234, *Rhizobium etli* and *Bradyrhizobium* spp. (Cardenas et al. 1995; Hussain et al. 1999; Banba et al. 2001; Schumpp et al. 2009; Bek et al. 2010). Traditionally these host/non-host relationships were described in the so-called cross-inoculation groups composed of legumes and their compatible rhizobia. However, this catalogue system is inconsistent and far from a perfect reflection of the host/non-host relationships as elucidated using molecular methods.

Ongoing efforts to fine-tune our understanding of host-microbiont specificity and the recognition process at the molecular level discovered a two-way signal exchange as one of the central components of recognition. Rhizobial NodD proteins mediate host recognition by interacting with specific flavonoids or isoflavonoids secreted from host roots (Peters et al. 1986; Spaink et al. 1989). Flavonoid-activated NodD promotes transcription of bacterial *nod*-genes involved in synthesis and secretion of lipochitin-oligosaccharides (Nod-factors). These molecules serve as major bacterial signals detected by the legume host (Mulligan and Long 1985; Spaink et al. 1991; Truchet et al. 1991). This signalling mechanism was elegantly demonstrated using two bacteria, *R. etli* and *M. loti*, from different cross-inoculation groups nodulating bean and *Lotus*, respectively. Both strains synthesise Nod-factors with the same structure. Artificially bypassing the flavonoid NodD activation by expression of a constitutive *nodD* transcriptional activator (FITA) in these rhizobial strains extended their host range beyond their cross-inoculation group to encompass both bean and *Lotus corniculatus* (Cardenas et al. 1995).

3 Nod-Factor Perception and Its Role in Host Determination

In legumes, the lipochitin-oligosaccharide Nod-factors of compatible bacteria initiate a signal cascade leading to nodulin gene activation, root cortical cell dedifferentiation and development of the root nodule (Spaink et al. 1991; Truchet et al. 1991; Hoegslund et al. 2009). The length of the Nod-factor carbohydrate moiety, the size and degree of saturation of the acyl chain and substitutions of the reducing and non-reducing glucosamine residues are characteristic for each rhizobial species. It is these structural features that determine whether the Nod-factor is perceived by the plant (Lerouge et al. 1990; Spaink et al. 1991; Truchet et al. 1991). *Mesorhizobium loti* strain R7A produces Nod-factors with pentameric GlcNAc backbones. At the reducing end moiety there is a 4-*O*-fucose with either an acetyl group or a proton in position 3 or 4. The nonreducing moiety is N-methylated and

N-acylated (*cis*-vaccenic acid or stearic acid), and a carbamoyl group is present in position 3 (Lopez-Lara et al. 1995; Bek et al. 2010). Phenotypic characterisation of mutants of *M. loti* strain R7A show an unchanged infection process and nodule formation in the absence of the N-Methyl and the carbamoyl substitutions. In contrast, the presence of the N-acetylated fatty acid was found to be crucial for nodulation. The acetylated fucosyl group was important for effective Nod-factor signalling and absence of this group led to host dependent nodulation phenotypes when comparing the four *Lotus* species *L. japonicus*, *L. filicaulis*, *L. corniculatus* and *L. burttii* (Rodpothong et al. 2009).

Perception of Nod-factor in *L. japonicus* is mediated by receptor kinases containing LysM modules in their extracellular domains. The two receptor kinases perceiving the Nod-factor signal, NFR1 and NFR5, are predicted to have a topology where single pass transmembrane domains anchor LysM containing extracellular domains and intracellular serine/threonine protein kinases (Madsen et al. 2003; Radutoiu et al. 2003). Combining this prediction with genetic evidence, a heteromeric receptor complex composed of NFR1 and NFR5 was proposed to initiate signal transduction following perception of a correctly decorated Nod-factor (Radutoiu et al. 2003). Biochemical studies have subsequently demonstrated NFR1 to be a dual specificity kinase capable of autophosphorylation and phosphorylation of NFR5 (Madsen et al. 2011). In contrast, no kinase activity could be detected for NFR5, which has domain features characteristic for pseudokinases. Membrane localisation and interaction between NFR1 and NFR5 was shown in vitro by enrichment in purified membrane fractions and in vivo through bimolecular fluorescence complementation (BiFC) in *Nicotiana benthamiana* and leek cells (Madsen et al. 2011).

The structural similarity between Nod-factors and chitin, a carbohydrate synthesised by different plant pathogens such as fungi, nematodes or insects, raise the possibility of an evolutionary link between chitin and Nod-factor perception, and thus between symbiotic and pathogenetic interactions. In support of this hypothesis, the *Arabidopsis* chitin receptor CERK1, which was shown to be involved in the perception of fungal pathogens (Miya et al. 2007; Wan et al. 2008), shows high sequence and structural similarity to the NFR1 protein (Radutoiu et al. 2003; Miya et al. 2007; Wan et al. 2008). Subsequent functional studies of the kinase domains further supported this close relationship. Exchange of the NFR1 kinase domain with the corresponding CERK domain and substitution of a portion of the αEF helix in CERK1 with the YAQ amino acid sequence from NFR1 reconstituted symbiotic signalling. Transgenic *L. japonicus nfr1* mutant plants carrying this swap construct were functionally complemented and able to develop nodules with *M. loti* (Nakagawa et al. 2011).

A deeper understanding of the host/non-host relationship between rhizobia and legumes was obtained by transforming the *L. japonicus* NFR1 and NFR5 genes into *M. truncatula*. Using this approach it was shown that NFR1 and NFR5 receptors act in concert as host determinants, transforming the non-host *M. truncatula* into a host able to recognise and be infected by *M. loti* and an engineered *Rhizobium leguminosarum* DZL producing a Nod-factor substituted with an acetylated fucosyl

on the reducing moiety (Radutoiu et al. 2007). Recognition of these normally non-compatible bacteria triggers root cell dedifferentiation, redifferentiation and initiation of nodule organogenesis as well as infection thread formation. This extended NFR1 and NFR5 mediated signal cascade is dependent on both Nod-factor synthesis, as shown with the *M. loti nodC* mutant, and structure as shown by the longer infection threads obtained with *R. leguminosarum* DZL, respectively. In line with these results it was shown that the *L. japonicus* NFR1 and NFR5 proteins also act in concert if introduced into *Lotus filicaulis*. Transgenic roots of this species expressing the *L. japonicus* receptor molecules can be nodulated by the *R. leguminosarum* DZL strain, which can nodulate *L. japonicus*, but is otherwise unable to nodulate wild type *L. filicaulis* roots (Radutoiu et al. 2007).

4 The Role of LysM Domains

Our understanding of the mechanisms involved in Nod-factor perception was further refined through domain swap experiments using receptors from *L. japonicus* and *L. filicaulis*. Initially it was observed that *L. filicaulis,* in contrast to *L. japonicus*, did not develop root nodules after inoculation with the *R. leguminosarum* DZL strain (Pacios-Bras 2003). Analysis of transgenic plants subsequently traced this inability back to variations in the amino acid composition of the NFR1 or NFR5 receptors between *L. japonicus* and *L. filicaulis* (Radutoiu et al. 2007). Domain swaps combined with substitutions of single amino acids were then used to show that specific recognition of the DZL Nod-factor relied on the LysM containing domains of NFR1 and NFR5 and that the LysM2 domain of NFR5 played a major role in discriminating *M. loti* and *R. leguminosarum* DZL Nod-factors in *L. filicaulis*. A leucine adjacent to a putative Nod-factor binding groove located between the first β-strand and first helix of the *L. japonicus* NFR5 LysM2 domain, a position filled by a lysine in *L. filicaulis*, was found to be largely responsible for the recognition of the Nod-factor synthesised by the DZL strain (Radutoiu et al. 2007). However, presence of three LysM domains in the NFR5 and NFR1 receptors (Madsen et al. 2003; Radutoiu et al. 2003; Arrighi et al. 2006), suggests the involvement of more than one LysM domain in Nod-factor perception. Two lines of evidence supports this notion: (1) the non-nodulation phenotype caused by an amino acid substitution in the LysM1 domain of the *M. trunctula* homolog of NFR5 called NFP (Arrighi et al. 2006) and (2) the involvement of LysM1 of the pea SYM37 NFR1-like receptor in distinguishing "European" and "Middle East" *R. leguminosarum* bv. *viciae* strains (Zhukov et al. 2008).

Further insight into the functional role of individual amino acids in Nod-factor perception was obtained in a domain swap study using the extracellular domains of NFR1 and NFR5 from a more distantly related species, *Lotus pedunculatus*. This *Lotus* species is normally nodulated by a *Bradyrhizobium* spp. strain producing a Nod-factor with an additional carbamoyl group at the non-reducing moiety (Bek et al. 2010). It was found that the combined amino acid differences of the NFR1 and NFR5

extracellular domains of *L. japonicus* and *L. pedunculatus* were not influencing the recognition of the Nod-factor substituted with one or two carbamoyls at the non-reducing end. Considering that a high number of amino acid variations is found between *L. japonicus* and *L. pedunculatus* NFR proteins, this suggests the involvement of only a small fraction of amino acids in deciphering Nod-factor structure.

5 Signal Transduction and Signalling Triggered by the NFR Receptors

In *L. japonicus* signal transduction following NFR-mediated Nod-factor perception is shared with the arbuscular mycorrhizal symbiosis. Common symbiotic signalling components include at least eight genes encoding the leucine-rich repeat RLK SYMRK (Stracke et al. 2002), the cation channels CASTOR and POLLUX (Imaizumi-Anraku et al. 2005), the nuclear pore proteins NUP133 (Kanamori et al. 2006), NUP85 (Saito et al. 2007) and NENA (Groth et al. 2010), the Ca^{2+}/calmodulin dependent kinase CCaMK (Tirichine et al. 2006) and the nuclear protein CYCLOPS (Yano et al. 2008). Analysis of *L. japonicus* mutants has shown that the LysM containing Nod-factor receptor(s), SYMRK, CASTOR and POLLUX, and the nuclear pore proteins are required for the induction of calcium oscillations, one of the earliest physiological responses detectable in root hairs after exposure to purified Nod-factor (Miwa et al. 2006; Saito et al. 2007). These calcium oscillations are thought to be interpreted by the *CCaMK* encoded Ca^{2+}/calmodulin dependent protein kinase (Miwa et al. 2006; Tirichine et al. 2006). A function in cross signalling between infection and organogenesis was proposed for CYCLOPS (Madsen et al. 2010), a direct interactor of CCaMK (Messinese et al. 2007; Yano et al. 2008). Downstream of the shared symbiosis pathway, putative transcription factors encoded by *Nin* (Schauser et al. 1999), *Nsp1* and *Nsp2* (Heckmann et al. 2006), and a member of the ERF family (Asamizu et al. 2008; Middleton et al. 2007) are required for regulation of nodule expressed genes and initiation of nodule organogenesis. One of these transcripion factors, NSP2, has been proposed to also contribute to arbuscular mycorrhizal symbiosis in *M. truncatula* (Maillet et al. 2011). This implies the interesting possibility that common symbiotic signalling could include the level of transcription-factor mediated downstream gene activation.

Formation of nodule primordia involves dedifferentiation and reactivation of cortical root cells. A combination of gain and loss of function mutants have shown that cytokinin signalling through the *Lhk1* cytokinin receptor is necessary and sufficient for the formation of nodule primordia (Murray et al. 2007; Tirichine et al. 2007). The phenotype of loss of function mutants (*hit1*) includes a drastic reduction in the number of primordia initiated, suggesting that cytokinin signalling is important for activation of the cell cycle in inner cortical foci that form the starting point of nodule organogenesis (Murray et al. 2007). This phenotype complies with

the detection of increased expression of a cytokinin reporter gene in the early phases of nodulation (Lohar et al. 2004). In the dominant *snf2* mutants encoding a gain of function LHK1 cytokinin receptor, the opposite phenotype is observed. *snf2* mutants develop root nodules spontaneously, i.e. in the absence of any rhizobia or Nod-factor signalling (Tirichine et al. 2007). This demonstrates that cytokinin signalling can induce the nodule meristem directly, presumably by activation of the cell cycle in distinct cell foci along the root. Both of these *Lhk1* mutants therefore affect the developmental events in the root cortex, suggesting that cytokinin is a component of the secondary epidermal-cortical signalling triggered by Nod-factor perception. The epidermal developments like root hair deformation and infection thread formation appear to be mainly indirectly affected by cytokinin signalling. In *hit1* loss of function mutants, hyperinfection in the form of an increased number of root hair infection threads are observed, and these infection threads are restricted in their progression into the cortex (Murray et al. 2007). This suggests that nodule primordia are involved in regulating infection thread formation through a negatively acting mechanism, and are necessary for their controlled progression into the inner cortex. The disruption of infection thread progression into the cortex in the *hit1* loss of function mutant further indicates a positive role for cytokinin signalling. Cytokinin is thus a candidate signal for the coordination of epidermal and cortical processes of infection and primordium formation, respectively.

6 Cell to Cell Recognition Mechanisms Regulate Bacterial Entry

Early investigations suggested plant lectin binding of bacterial specific extracellular carbohydrates to be the major determinants of plant-rhizobium host range (Bohlool and Schmidt 1974). Subsequent experiments demonstrated binding of legume lectins to rhizobial surface polysaccharides (Bourne et al. 1994), and indicated that lectins facilitate rhizobial attachment to the root hair tips by binding surface polysaccharides and thereby increasing the local Nod-factor concentration above the required threshold for nodule initiation (van Rhijn et al. 2001). Studies performed with bacterial mutants showed that lectin mediated recognition is particularly important for infection thread progression through cortical cell layers (van Rhijn et al. 1998). Rhizobial host range extension as a result of lectin gene transfer from pea or soybean into clover, alfalfa or *L. corniculatus* appears also in most cases to be dependent on the Nod-factor structure and/or bacterial exopolysaccharides (van Rhijn et al. 1998, 2001). The mechanism and contribution of plant lectins to the infection process is therefore awaiting further investigation. For a recent review summarising the role of lectins in plant-microbe interactions see De Hoff et al. (2009).

The precise role of the different rhizobial surface polysaccharides for symbiotic partner recognition of bacteria and/or for the complete infection of the plant root tissues is on the other hand, well documented through genetic analysis of rhizobia.

cgs mutants of *M. loti* R7A or strain Ayac 1 BII that are deficient in the synthesis of cyclic ß-glucan induced the formation of empty nodules on *L. japonicus* (Kelly, personal communication; D'Antuono et al. 2005, 2008). Infection thread development was impaired indicating a role for cyclic ß-glucan during infection thread formation or progression. The lipopolysaccharide (LPS) deficient *lps* mutants of *M. loti* tested on *L. japonicus* so far were only marginally affected in establishing symbiosis, and the phenotypes observed are mainly associated with strain competitiveness (Kelly, personal communication; D'Antuono et al. 2008). Further studies are required in order to determine the influence of LPS on symbiosis in *L. japonicus*, which in light of the recent report of nitric oxide (NO) release in response to purified LPS may be complex (Murakami et al. 2011). Exopolysaccharides (EPS) appear to be more important for infection of *L. japonicus*. Nodulation of *L. japonicus* by *eps* mutant strains defective in genes that are involved at mid-late stages of EPS biosynthesis (*exoU*, *exoO*, *exoK* and *mlr5265*) was severely affected. These mutant bacteria induced the formation of small white bumps devoid of bacteria. However, an EPS mutant strain (*exoB*) disrupted in a very early stage of EPS biosynthesis forms nitrogen-fixing nodules indistinguishable from those induced by wild-type *M. loti*. Visualisation of the *exoU* mutant bacteria during the infection process revealed that the EPS-deficient strain was disrupted in its ability to induce normal infection thread formation. The host plant may therefore be able to decipher the EPS structure presented by the invading bacteria most likely through a specialised EPS receptor (Kelly, personal communication). A specific role for bacterial EPS is further supported by an earlier study showing that several *eps* mutant strains of *M. loti* strain PN184 lose compatibility with the host *Leucaena leucocephala*, while retaining full compatibility with a second host, *L. pedunculatus* (Hotter and Scott 1991).

Strains of *M. loti* possess either type III or type IV (e.g. MAFF 303099 and R7A, respectively) secretion systems that in several cases have been shown to deliver effector proteins into cells of eukaryotic hosts and to be required for virulence of many Gram-negative bacterial pathogens. In the *M. loti* strain R7A, expression of genes encoding components of the type IV secretion system is specifically regulated in a symbiosis-dependent manner and linked to the presence of the host (Hubber et al. 2004, 2007). Inactivation of the type III secretion system in the *M. loti* strain MAFF affects symbiosis neutrally, positively or negatively dependent on the *Lotus* species tested (Sanchez et al. 2009; Okazaki et al. 2010). *L. japonicus* was unaffected, while *L. filicaulis* and *L. coniculatus* subsp. *frondosus* formed fewer root nodules in the absence of the secretion system. In contrast, there was a strong negative effect on infection of *L. halophilus*, which appears capable of detecting a small secreted bacterial effector protein and halting symbiosis development in response (Okazaki et al. 2010). Mutation of the gene encoding this protein reverts the phenotype to fully functional root nodules. Drastic phenotypic changes resulting from mutation of the type III and IV secretion system were found on the alternative host *L. leucocephala* (Hubber et al. 2004, 2007; Okazaki et al. 2010). Both the R7A and MAFF wild type strains of *M. loti* induced tumour-like nodules on this host. Inactivation of both the type III and IV protein effector secretion

systems in R7A and MAFF lead to the development of fully infected and functional root nodules. Recognition of effector proteins resulting in either suppression of defence responses or induction of the defence response following detection of secreted proteins as pathogen effectors could explain these opposite responses (Hubber et al. 2004, 2007). Interestingly, a gene encoding a Toll-interleukin leucine-rich repeat receptor (TIR-NBS-LRR) suggested to recognise a rhizobial protein effector was identified in soybean (Yang et al. 2010).

7 Invasion Through Root Hair Infection Threads

In *L. japonicus* the normal infection occurs through infection threads initiated in elongating root hairs of the susceptible region located just behind the root tip. Upon formation of infection pockets, the root hair cell wall dissolves and an infection thread is initiated by invagination and subsequent polar extension of the plasma membrane, which is accompanied by the deposition of new cell wall material (see Gage (2004) for a review). Inward growing infection threads progress through the root hair and are propagated through the cell layers by a reiterated cell autonomous mechanism. Such infection threads are only formed in the presence of rhizobial bacteria. Nod-factor alone elicits the earliest detectable responses such as membrane depolarisation, ion fluxes across the membrane, Ca^{2+} spiking and cellular alteration in actin and microtubule organisation, but not infection threads (Weerasinghe et al. 2003, 2005; Vassileva et al. 2005; Miwa et al. 2006). Interestingly, a class of mutants characterised by a lack of or reduced infection thread progression have been identified (Schauser et al. 1998; Lombardo et al. 2006; Yano et al. 2006; Murray et al. 2007). In these mutant lines the infection threads were typically arrested either within root hairs/epidermis or within the first cortical cell layers. Furthermore, mutant lines were also identified where infection thread formation was not followed by the release of bacteria into cells of the nodule primordium (Imaizumi-Anraku et al. 1997).

Mutant lines known to affect infection thread formation include *nin* (Schauser et al. 1999), *nsp1* and *nsp2* (Heckmann et al. 2006), *cerberus* (Yano et al. 2009), *nap1* and *pir1* (Yokota et al. 2009), *alb1-1* (Imaizumi-Anraku et al. 2000), *crinkle* (Tansengco et al. 2003), as well as *itd1*, *itd3* and *itd4* (Lombardo et al. 2006). Around half of these have been characterised. A functional *Nin* gene is required for infection thread initiation and for restricting root hair deformation, as well as restricting the size of the infection zone. The domain structure of the NIN protein suggests a function as transcriptional regulator, and the mutant phenotype demonstrates an essential role for the protein in both infection thread formation and organ initiation (Schauser et al. 1999). The phenotype and the transcripts affected in this mutant background also suggest the involvement of NIN in both positive and negative regulation and coordination of infection and organogenesis (Schauser et al. 1999; Hoegslund et al. 2009). Like NIN, NSP1 and NSP2 appear to be involved in activating the gene expression required for infection thread

formation. The *L. japonicus* gene set controlled by these regulators has so far not been identified, but detailed analysis of the putative *M. truncatula* orthologs of NSP1 and NSP2 suggests that they act as a transcription factor complex activating several downstream genes, including *NIN* and the symbiosis-induced gene ENOD11 (Hirsch et al. 2009). *L. japonicus* plants carrying a mutant *nap1* or *pir1* allele had a significantly diminished ability to capture bacteria within infection pockets and initiating infection threads (Yokota et al. 2009). The rare infection threads that formed disintegrated, and infection threads extending to the base of the root hair cells were only occasionally observed. Lack of infection threads in the root cortex further suggested that NAP1 and PIR1 were essential for the progression of the infection process. Further characterisation showed that the *Nap1* and *Pir1* genes are essential for establishing the actin organisation in root hairs (Yokota et al. 2009). Molecular analysis revealed the putative *Arabidopsis* orthologs of these proteins, NAP1 and PIR1, as likely components of the SCAR/WAVE complex that activates the ARP2/3 complex, which binds pre-existing actin polymers and nucleates new actin filaments (Brembu et al. 2004; Deeks et al. 2004; Li et al. 2004). The role of the *Cerberus* encoded ubiquitin-E3-ligase for infection thread formation is less clear, but its requirement suggests that there is a need for clearing the root hair of particular proteins in order to secure infection thread progression (Yano et al. 2009). A membrane raft associated remorin belonging to a subgroup found so far only in plants of the Rosid I clade, which all nodulators are part of, is upregulated during nodulation (Lefebvre et al. 2010). In *M. truncatula* this remorin interacts with symbiotic receptors and localises to infection thread- and symbiosome-membranes (Lefebvre et al. 2010). Functionally the remorin was linked to a role in bacterial release and endocytosis, and symbiosis was impaired in remorin mutant plants. So far remorin mutants have not been isolated in *L. japonicus*, but it will be interesting to investigate the role of this putative membrane anchor for the NFR receptors in determinate nodulation. Similar roles have been suggested for flotillins (Haney and Long 2010) and Rab proteins (Limpens et al. 2009) within indeterminate nodules of *M. truncatula*. Different *Rab* protein encoding mRNAs have been shown to be upregulated in *L. japonicus* nodules (Borg et al. 1997), and analysis of these, as well as of *L. japonicus* homologs of remorins and flotillins may further contribute to a better understanding of infection during determinate root nodule formation.

8 Parallel Signalling Pathways Regulate Root Hair Infection and Nodule Development

Bacterial infection of legume root nodule primordia is tightly controlled and closely synchronised with the progressing primordial cell divisions and organ development. This coordination has long prevented the separation of the molecular mechanisms underlying these two developmental processes and has limited the

identification of plant genes controlling the bacterial infection process, but not nodule organogenesis. As a consequence the genetics of infection thread development is fairly undescribed and among the genes defining this pathway, only *Nap1*, *Pir1* and *Cerberus* have so far been characterised at the molecular level (Yano et al. 2009; Yokota et al. 2009). Most of the Nod-factor signal transduction pathway mutants mentioned above suffer from simultaneous absence or severe impairment of both organ formation and infection thread development. This has made the dissection of direct and indirect mutational impact on the two processes very difficult. However, combining gain of function mutations with loss of function mutations in trangenes or double, triple and quadruple mutants opened new possibilities for assessing the role of the genes inactivated by loss of function mutations (Hayashi et al. 2010; Madsen et al. 2010). The autoactive versions of CCaMK and LHK1 encoded by the *snf1* or *snf2* alleles, respectively, were key to this approach. Both *snf* mutants form spontaneous nodules independent of bacterial presence or infection (Tirichine et al. 2006, 2007). Exploiting the ability of these gain of function alleles to activate the developmental processes from downstream positions defined the backbones of two parallel pathways facilitating (1) infection thread formation and (2) root nodule organogenesis (Hayashi et al. 2010; Madsen et al. 2010). Furthermore, the approach revealed cross-signalling functions between these two pathways for some of the examined genes. It was shown that the LRR receptor kinase SYMRK, the nucleoporins NUP133 and NUP85 and the cation channels CASTOR and POLLUX, all involved in signal transduction down-stream of Nod-factor perception, were dispensable for root hair infection thread development and invasion of nodule primordia in a *snf1* genetic background. Since inactivation of the LRR receptor kinase SYMRK, the nucleoporins and the cation channels results in absence of calcium spiking (Miwa et al. 2006), these results further indicate that calcium spiking, apart from activation of CCaMK, is dispensable for infection thread formation (Madsen et al. 2010).

On the other hand, the NFR1 and NFR5 Nod-factor receptors were required for root hair infection thread initiation, and the NAP1 and PIR1 proteins mediating actin rearrangement together with the CERBERUS ubiquitin E3 ligase were required for infection thread progression (Madsen et al. 2010). These observations support an early branching of the Nod-factor signal transduction pathway. The NIN, NSP1 and NSP2 transcriptional regulators were required for both infection thread formation and organogenesis, supporting a simultaneous or sequential role of these proteins in both of these processes. The absence of infection in *cyclops snf1* double mutants (Madsen et al. 2010) along with the reported protein-protein interaction between CCaMK and CYCLOPS (Messinese et al. 2007; Yano et al. 2008), indicated a role for CYCLOPS in cross signalling between the organogenic and infection pathways (Madsen et al. 2010).

Nod-factor induced root hair deformation, Ca^{2+} influx and Ca^{2+} spiking preceding infection thread formation all require the initial NFR1 and NFR5 mediated perception event (Radutoiu et al. 2003; Miwa et al. 2006). The relation between these phenomena is however not yet fully clarified. Ca^{2+} spiking following application of Nod-factors is not required for root hair deformation (Miwa et al. 2006)

and the presumed Ca^{2+} dependent activation of CCaMK as mimicked by the *snf1* mutation seems insufficient for root hair infection thread formation (Madsen et al. 2010). Separation of root hair deformation and influx was also implied by the observation that three orders of magnitude lower Nod-factor concentration was sufficient to induce root hair deformation compared with that required for the Ca^{2+} influx (Radutoiu et al. 2003; Miwa et al. 2006). This could suggest that the NFR receptor dependent Ca^{2+} influx observed in root hairs at high Nod-factor concentration may be a prerequisite for root hair infection thread formation. So far these observations are consistent with the Ca^{2+} responses occurring in parallel with the NAP1 and PIR1 induced cytoskeletal changes, although the possibility of a low level or localised change in Ca^{2+} cannot be excluded.

9 Alternative Infection Mechanisms: Crack Entry

Microscopical surveys of infection in legume species representing different clades of the legume family have revealed the existence of a number of alternative infection modes leading to nitrogen fixing root nodules (recently reviewed in Held et al. (2010)). However, with only a few exceptions like *Sesbania rostrata* (Goormachtig et al. 2004), generally only one infection mode is found in an individual species. *L. japonicus* is, for example, infected via intracellular root hair infection threads (Szczyglowski et al. 1998; Schauser et al. 1999; Lombardo et al. 2006), while peanut is infected via an intercellular crack entry process (Fabra et al. 2010). One of the surprising outcomes of the experiments combining gain of function and loss of function mutations was the discovery of two additional, although less effective, intercellular infection modes in *L. japonicus* (Madsen et al. 2010). Loss-of-function alleles of particular genes also indicated the presence of a crack entry based infection mode (Karas et al. 2005; Groth et al. 2010), and mutational abrogation of both root hair and cortical infection thread formation revealed a further mode of infection of presumably more ancient evolutionary origin (Madsen et al. 2010).

A mechanism enabling intercellular infection and formation of trans-cellular infection threads within the root nodules was found to operate in the absence of functional NFR1 and NFR5 receptors. Despite the independence from these proteins known to be required for Nod-factor perception at early stages, the formation of trans-cellular infection threads in nodules did depend on bacterial production of intact Nod-factors, and *nodC* mutants of *M. loti* could not induce their development (Madsen et al. 2010). It is yet unclear which molecules are involved in the evident Nod-factor perception in the root cortex. Receptor kinases belonging to the *L. japonicus* LysM receptor-like kinase (*Lys*) family, which includes NFR1 and NFR5, are possible candidates (Lohmann et al. 2010). Of these, *Lys* genes that are expressed predominantly in root and nodule tissues, namely *Lys2*, *Lys3*, *Lys7*, *Lys12*, *Lys15* and *Lys20* (Lohmann et al. 2010), are the most likely to be involved. A role of alternative epidermal or cortical Nod-factor receptors is further supported

by observations in the tropical legume *S. rostrata*. Root hair infection was strictly dependent on an intact Nod-factor structure, while root-hair independent crack entry at lateral root bases was less stringently controlled in this species (D'Haeze et al. 2000; Goormachtig et al. 2004).

The downstream components involved in signalling upon Nod-factor perception by presumed cortical receptors are unknown. The complete absence of infection in *cyclops snf1* double mutants suggests that CCaMK activation followed by signalling through a CCaMK CYCLOPS complex is required for cortical infection thread formation (Madsen et al. 2010). In contrast, infection studies in recently identified mutants of the *L. japonicus* nucleoporin NENA suggest that crack entry and cortical infection thread formation can occur independent of CCaMK activation through Ca^{2+} spiking, or alternatively rely on rare activation events (Groth et al. 2010). In line with the former interpretation, crack infection of the outer cortex was retained in CCaMK knockdown-roots of *Sesbania* despite the loss of root hair infection and nodulation (Capoen et al. 2009).

10 Alternative Infection Mechanisms: Single Cell Peg Entry

Characterisation of synthetic mutants combining gain of function (*snf1*) and loss of function mutations lead to a remarkable and unexpected discovery of an infection thread independent single cell entry mechanism in *L. japonicus* (Madsen et al. 2010). This intracellular infection of individual host cells by a process best described as peg entry may constitute the ground state of bacterial invasion during evolution of nodulation, and may allow for an analysis of the cardinal requirements distinguishing endosymbiotic co-existence from pathogenesis. Surprisingly, this entry mode was independent of both the NFR1 and NFR5 plant receptors and the rhizobial Nod-factor signal molecule (Madsen et al. 2010). In a *snf1* gain of function genetic background, a bacterial *nodC* mutant unable to produce Nod-factors was capable of infecting *nfr1 nfr5* single and double mutants although at a 20–100 fold reduced efficiency compared to normal root hair invasion. Infection threads were not observed, but symbiosomes were present in the infected cells, indicating that intercellular infection followed by endocytosis occurred in the absence of Nod-factor and the NFR1 and NFR5 Nod-factor receptors (Madsen et al. 2010). Such a capacity for direct intercellular infection could constitute the ancient invasion path that evolved at the emergence of the legume family or possibly at the emergence of the eurosid I clade containing all nodulating plants. Continued division of the primary infected single cells would lead to the fully infected Nod-factor independent nodulation observed in *Aeschynomene* type nodules (Sprent 2007). Nod-factor independent stem nodulation of *Aeschynomene* species (Giraud et al. 2007) appears to be a rare exception, however, and in many legumes the subsequent evolutionary step may have been the Nod-factor and cortical Nod-factor receptor dependent crack entry and subsequent cortical infection thread propagation of invasion as seen in *S. rostrata* (D'Haeze et al. 2000). The

Nod-factor dependent crack entry without infection threads and the fully infected nodules in *Arachis* and *Stylosanthes* (Noti et al. 1985; Wilson et al. 1987; Boogerd and van Rossum 1997) can be considered a variant of this infection mode. Fixation thread symbiosis, where rhizobia are not released from the infection thread, as seen in many legume trees and in *Parasponia* (Trinick 1979; Sprent 2007), is another variant. Interestingly, the Gram-positive *Frankia* bacteria inducing actinorhizal symbiosis in non-legumes are contained in fixation threads comparable to those seen in legumes belonging to the oldest legume subfamily, the *Caesalpinioideae* (Pawlowski and Bisseling 1996). Bacterial invasion via crack entry followed by the formation of either fixation threads or infection threads with bacterial release into symbiosomes in different members of the caesalpinoid genus *Chamaecrista*, support the notion of crack entry as a basal feature (Naisbitt et al. 1992; Sprent 2007). The most highly evolved state suggested by the differential signalling and genetic requirements of entry modes revealed in *L. japonicus* is root hair infection. This infection mode required Nod-factor, epidermal NFR1 and NFR5 receptors, and is predicted to also involve cortical Nod-factor receptors with less stringency than NFR1 and NFR5. Overall this model predicts an evolutionary process leading from pathogen-like bacterial invasion between cells to the more intimate containment of – and coexistence with – symbiosomes inside plant cells.

Taken together, the advanced steps of bacterial release seen in the more recently evolved legume hosts containing symbiosomes inside their cells have provided these plants with a tighter and more selective control of bacterial proliferation and nutrient exchange within the host. Similarly, the root hair infection mode seen in many evolutionarily young legume groups allows for a tighter control of bacterial passage through the epidermis than crack entry mechanisms. Observations of infection modes in a wide selection of legumes belonging to different subfamilies also indicate that alternative invasion modes have been maintained during evolution and that they are not mutually exclusive. Presence of different infection modes in *Sesbania* (Goormachtig et al. 2004), *Chamaecrista* (Sprent 2007) and both a Nod-factor dependent and a Nod-factor independent mechanism for nodulation in *Aeschynomene sensitiva* (Giraud et al. 2007) is in accordance with this scenario. Further comparison of the infection thread and direct infection pathway(s) seems to provide a prime opportunity to assess the basal genetic components involved in the release and endocytosis of bacteria.

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Signalling and the Re-structuring of Plant Cell Architecture in AM Symbiosis

Andrea Genre

Abstract Arbuscular mycorrhizas are widespread and ancient plant symbioses that were already established by the first land plants when they abandoned the water environment. Arbuscular mycorrhizal fungi scavenge mineral nutrients and water from the soil improving the overall plant fitness, receiving in exchange carbohydrates that are indispensable to complete their life cycle. In spite of their importance in natural and agricultural ecosystems, many biological aspects of these interactions are still partially obscure, especially concerning the early stages of symbiosis establishment which involve a signal exchange between the partners. Nonetheless, recent advancements have started to shed light on plant–fungus signalling mechanisms and their relation with the cell responses that culminate in fungal accommodation in the root cells. This chapter is focused on such advances and the new views that they have suggested.

1 Introduction

Arbuscular mycorrhizas (AM) are considered to be the most ancient plant symbiosis. Fossil data report the presence of arbuscules inside the tissues of some of the first plants that abandoned the water environment to start dry land colonisation (Remy et al. 1994). The ability to scavenge mineral nutrients and water from humid soils has likely been the key to the success of AM fungi, and hence of the plants that were hosting them. Present-day soils retain their nutrients as strongly as those prehistoric shores (Leeper 1952). It is therefore not surprising that AM interactions are still so popular in the plant kingdom. Eighty to ninety percent of all plant species interact in natural and agronomical environments with AM fungi (Smith and Read 2008).

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In spite of its widespread distribution and basic importance in plant nutrition, our knowledge of several biological aspects of the AM symbiosis is still patchy, especially when compared to other plant interactions, such as those with pathogenic fungi or oomycetes. Beside the economical aspects (defeating a pathogen has been so far much more profitable than exchanging chemical fertilisers for symbiotic fungi), several biological reasons account for this knowledge gap. First of all, AM fungi, which all belong to the small phylum of Glomeromycota, are rather unique organisms, probably also as a consequence of their long co-evolution with plants. AM fungi cannot reproduce in the absence of their hosts, they are strictly asexual and their huge spores (up to 500 μm) may contain a mixed population of thousands of nuclei, bearing different sets of genes (Hijri and Sanders 2005; Sanders and Croll 2010). The very concepts of individual, species and cell are difficult to apply to these fungi (Pawlowska and Taylor 2004), which are aseptate and even anastomose with each other mixing their respective nuclear populations through strong cytoplasmic streams (Croll et al. 2009). Summing up so many unusual features, AM fungi clearly do not fit the definition of a model organism for laboratory investigations. For example, classic genetic studies are totally unsuitable for these fungi (Vandenkoornhuyse et al. 2001). On top of that, rather than developing on a leaf – in full view of an attentive observer – AM interactions take place underneath the soil surface, strongly hampering our chances to understand most of the early steps of their development, including signal exchanges between the plant and fungus. These have only recently been approached thanks to important technical advancements, such as the ability to obtain and follow the symbiosis development inside a Petri dish (Bécard and Fortin 1988; Cano et al. 2008), and have rapidly attained the mainstream of AM research.

After a quick overview of the root colonization process by AM fungi, this chapter will focus on the recent advancements in the study of plant–fungus signal exchanges and their relation with the plant responses that culminate in fungal accommodation inside the root cells. Compared to other plant–microbe interactions, the resulting picture is still missing many major elements, but new chances can now be taken to peek into the biological processes that control the establishment of AM symbiosis.

2 The Process of Root Colonization

The development of root colonization by Glomeromycota is largely conserved throughout host plant taxa. Under favourable environmental conditions, the large asexual spores of AM fungi produce germination hyphae that can only grow for a few days in the absence of a host plant (asymbiotic phase). This short mycelium is only fed by the spore, as it is unable to uptake carbon from the soil organic matter (Leight et al. 2009), and its life span is limited to a few days, after which the hyphae stop growing and retract their cytoplasm into the spore, in view of a new germination event. By contrast, as soon as AM hyphae reach the vicinity of a host root

(presymbiotic phase), they visibly change their developmental program and branch repeatedly (Buee et al. 2000). This phase, when the plant and fungus perceive each other, is followed by direct plant–fungus contact, which associates with the setup of novel developmental and cellular modifications in both partners. The hyphae of glomeromycetes normally explore a stretch of the contacted root before changing their growth pattern to form highly branched, swollen and flattened hyphal protrusions that strongly adhere to the wall of epidermal cells (Smith and Read 2008). Such a structure is known as a hyphopodium (Bastmeyer et al. 2002) and its adhesion to the atrichoblasts of the root epidermis marks the initiation of the symbiotic phase of the interaction. Root epidermal cells in turn respond to hyphopodium contact by repositioning their nucleus and remodelling their cytoplasm, in preparation for fungal penetration (Genre et al. 2005). In fact, the entry of AM fungi in the plant cell lumen implies the development of a novel cellular compartment, the symbiotic interface. This structure, of plant origin, is composed of a thin layer of cell wall materials in direct contact with the fungus, and an invagination of the host plasma membrane – named the perifungal membrane (Bonfante 2001). The interface compartment envelopes every intracellular hypha, from the simplest, straight hypha crossing an epidermal cell to the highly branched arbuscule filling up most of a cortical cell volume. In most cases fungal colonisation proceeds intracellularly across the epidermal and outer cortical cells. By contrast, once the hyphal tips have reached the inner cortex they can also develop along the intercellular spaces of the cortical parenchyma (Dickson 2004). This intercellular mode of growth is particularly common in legumes, and in general in those plants (e.g. *Arum* sp.) whose root anatomy presents such extensive apoplastic channels. In these cases, most of the arbuscules develop from terminal hyphal branches, whereas files of intercalary arbuscules originate when inner cortex colonisation mostly proceeds from a cell to the next with a so-called intracellular mode of growth (e.g. carrot or *Paris* sp.).

Arbuscules – the structures that give AM interactions their name – originate from the repeated branching of an intracellular hypha. Arbusculated cells are the site where symbiosis achieves its highest degree of complexity from a morphological and functional point of view. The symbiotic interface, lined by the periarbuscular membrane, envelopes each fine branch of the arbuscule and mediates nutrient – and likely signal – exchanges. Originally suggested by the very high surface-to-volume ratio achieved by the arbuscule recursive branching (Dickson and Kolesik 1999), the concentration of nutrient exchange in the arbuscules has recently found more direct evidence in the identification of plant phosphate and ammonium transporters that are mainly expressed in arbusculated cells (Javot et al. 2007; Balestrini et al. 2007; Guether et al. 2009) and localized on the periarbuscular membrane (Pumplin and Harrison 2009; Kobae and Hata 2010).

The establishment of a functional AM symbiosis therefore involves a progressive increase in the closeness of the interaction, from the exchange of long-range chemical signals in the rhizosphere to intimate intracellular association, when the plant and fungus share a single cell volume.

3 The Presymbiotic Dialogue

Increasing evidence has accumulated over the years concerning the exchange of chemical signals between the plant and fungus in advance of their first physical contact. A summary of the resulting picture is presented in Fig. 1. Keeping both partners informed on their proximity is an obvious advantage in preparation for direct interaction. In the case of AM, this is achieved through an apparently rough, but successful strategy: the perception of plant diffusible signals induces hyphal ramification within hours (Akiyama et al. 2005), while fungal proximity stimulates lateral root development in days (Olah et al. 2005). As a consequence, the chances for the hyphae and roots to eventually meet increase exponentially with reciprocal vicinity. Such presymbiotic developmental responses associate with metabolic, transcriptional and cellular responses, as part of an "anticipation program" that prepares both symbionts to a successful association (Paszkowski 2006a).

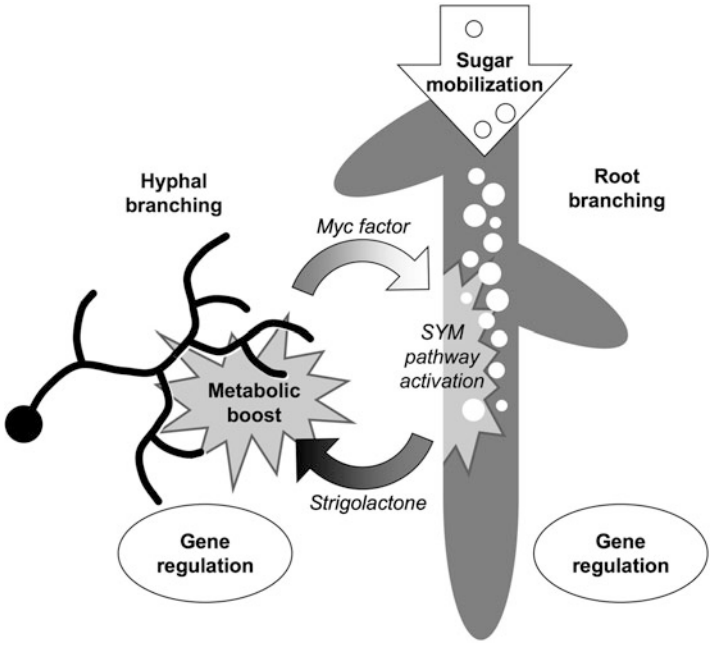

Fig. 1 Presymbiotic signalling. The scheme summarises the main responses observed in both the plant and fungus during the presymbiotic phase of an AM interaction. Strigolactones released by the host plant root activate fungal metabolism, stimulate hyphal branching and induce gene regulation. At the same time, the perception of a so-far unidentified Myc factor by the plant is mediated by the SYM transduction pathway and leads to gene regulation, starch accumulation in the root and the development of new lateral roots

3.1 Strigolactones: Eclectic Plant Messengers

Direct evidence for a chemotropic development has only been found for a few glomeromycetes (Sbrana and Giovannetti 2005), while Akiyama et al. (2005) have identified a strigolactone secreted by *Lotus japonicus* roots as the molecule responsible for stimulating such host-induced hyphal branching in AM fungi. Furthermore, *Sorghum* strigolactones have also been shown to boost AM hyphal growth and metabolism, and increase mitochondrial number and motility (Besserer and Puech-Pagès 2006; Besserer et al. 2008). Strigolactone instability in water and their continuous release from the root combine into a steep concentration gradient, which provides precise topological information about root localization and directly impacts the pattern of fungal development.

Interestingly, the biological activity of strigolactones goes beyond AM interactions, as confirmed by the fact that their production is not limited to AM host plants (Delaux et al. 2009): such carotenoid-derived molecules have in fact several and very diverse functions. Their principal role is likely to be hormonal. Being produced both in the root and shoot, strigolactones control shoot branching by inhibiting lateral bud development (Gómez-Roldán et al. 2008). Their role as rhizospheric messengers seems therefore to have originated secondarily, due to their continuous leakage from the roots. Not surprisingly, the strigolactone gradient is also exploited by plant parasites. Seed germination in parasitic plants such as *Striga* (Cook et al. 1966) is boosted by the presence of root-exudated strigolactones – whose name in fact derives from this very plant genus. Tomato plants bearing a mutation in a gene involved in strigolactone biosynthesis (*SlORT1*) are both resistant to the parasitic weed *Orobanche* and reluctant to AM colonization (Koltai et al. 2010). Nevertheless, Akiyama et al. (2010) have recently highlighted the fine structural requirements that specifically correlate with the activity of strigolactones in stimulating AM fungal branching versus parasitic plant seed germination. It therefore appears likely that, during the co-evolution of different organisms with plants, differential signalling mechanisms have tuned to specific forms of strigolactones.

In spite of the long studies carried out on parasitic plants, and the more recent investigations on AM fungi and epigeous plant tissues, one missing tile in this complex communication system is the identification of the proteins that mediate strigolactone perception. Two genes encoding for an F-Box protein and an α/β-fold hydrolase have recently been identified as potential strigolactone receptors in the shoot of rice (Ishikawa et al. 2005; Arite et al. 2009), and a 60 kDa strigolactone-binding protein has been isolated from *Striga hermontica* (Zwanenburg et al. 2009). Such studies may open the way to the identification of proteins with an analogous function in the fungus.

In conclusion, strigolactones are considered as the main actors in the plant-to-fungus communications that precede their physical interaction. We cannot anyway exclude that other factors are also involved. If we consider the relatively similar case of nitrogen-fixing root nodules, beside the acknowledged flavonoid-based signalling mechanism required for symbiosis establishment, a role has recently

been suggested for strigolactones also (Soto et al. 2010), thus introducing a possible crosstalk between the early events that control legume root symbioses.

3.2 The Elusive AM Fungal Signals

The perception of fungal diffusible signals by the host plants was demonstrated by several publications over the last years. Such signals are often referred to as "Myc factor" in analogy to the Nod factor produced by nitrogen fixing rhizobia (Kosuta et al. 2003), Maillet et al. (2011). Myc factor perception induces the first molecular and cellular responses in the host plants several hours before the development of hyphopodia on the root surface. The Myc factor diffuses in water solutions and can cross permeable membranes that prevent physical contacts between the two partners. In these conditions, fungal diffusible factors were shown to activate the expression of the early nodulin *MtEnod11* and other symbiosis-related genes that are also expressed during root colonization (Kosuta et al. 2003; Weidmann et al. 2004). More recently, a membrane steroid binding protein (*MtMSBP1*) and other plant genes have also been convincingly shown to be upregulated in the root tissues approached by the branching AM hyphae (Kuhn et al. 2010).

Beside the stimulation of lateral root development (Olah et al. 2005), another long-term host plant response to diffusible fungal signals was detected while investigating root starch content. A decrease in root starch level is normally associated with arbuscule development. By contrast, when *L. japonicus* plants were exposed for 1 week to AM fungal exudates or grown in the presence of a membrane to prevent fungal contact and colonization, starch accumulated in the root cortex and was stored there for at least 1 month (Gutjahr et al. 2009). Such a change in carbohydrate mobilisation can easily be interpreted as another element of the anticipation program, preparing the carbohydrate supplies that should normally be bound for the fungus.

Such studies demonstrate beyond question that host plants respond to diffusible AM fungal signals. Nonetheless, many aspects of the responsible signalling mechanisms remain unknown. First of all, the chemical structure of the Myc factor itself is currently under investigation, and we cannot exclude that several different molecules contribute to its biological activity, also based on the variety of plant responses observed. Under this respect, the recent identification, in AM fungal exudates, of lipochito-oligosaccharides with a structure and effects that are very similar to those of the rhizobial Nod factors, hints at a further level of crosstalk between the two symbioses (Maillet et al. 2011). On the other hand, the upregulation of a steroid-binding protein in response to diffusible AM fungal signals suggests the involvement of lipid-based signals (Kuhn et al. 2010).

The plant receptors involved in Myc factor perception are also unknown, but the availability of a few mutant lines affected in AM colonisation has allowed the identification of genes and proteins that have been positioned along a putative and only partially characterised signalling pathway.

4 The SYM Pathway

Most – although not all – of the responses described so far are in fact mediated by the activity of a few known gene products (Kistner et al. 2005). These are expressed in all AM host plants and are known as the "common" SYM genes (Oldroyd and Downie 2006), with reference to the dual role they have in legumes, in controlling both AM symbiosis and nitrogen-fixing nodulation (Parniske 2008). While, anyway, a few more genes have been identified in nodulation-specific branches of this pathway, including the Nod factor receptor-like kinases *LjNFR1/5* and *MtNFP*, no SYM pathway-related gene has so far been characterised whose activity is unique to AM interactions.

From a topological perspective (Fig. 2), our partial view of the SYM pathway shows a very marked directionality from the cell boundaries to the nucleus, which provides this hypothetical signal transduction pathway with sound rational support. *DMI2* (for *Doesn't Make Infection*), the receptor-like kinase of *Medicago truncatula* potentially contributing to Myc factor recognition is localised on the plasma membrane. Three versions of DMI2 homologs are known in angiosperms, and interestingly the shortest proteins from rice and maize (*SYMRK)* only recognise AM fungal signals, whereas the longest homologs, found in legumes, are involved in the perception of both Myc and Nod factors (Markmann et al. 2008).

All forms expose their kinase domain on the cytoplasmic side of the plasmalemma. The unknown substrate of this kinase is therefore supposed to be a free cytoplasmic protein. Either this substrate or an indirectly generated signal is probably reaching the nucleoplasm through nuclear pores. Two nucleoporins, *LjNUP133* (Kanamori et al. 2006) and *LjNUP85* (Saito et al. 2007), have in fact been described in *L. japonicus* and are indispensable for fungal accommodation and AM establishment. The fact that such nucleoporins, at least in animals, are not known to be directly involved in the canonical nuclear pore import/export mechanisms, leaves anyway the question open about their actual role within the SYM pathway (Parniske 2008).

Not surprisingly, the nucleus is the organelle where all the remaining SYM gene products concentrate. The putative cation channels identified as CASTOR and POLLUX in *L. japonicus* (Charpentier et al. 2008), homologs of *M. truncatula DMI1* (Ané et al. 2004), are localised on the nuclear envelope and it has been hypothesized that they could be the cargo of NUP133 and NUP85 (Parniske 2008).

The two last SYM genes involved in AM signalling both localise in the nucleoplasm. *MtDMI3* (Lévy et al. 2004) is a kinase whose activity is regulated by the binding of both calcium and calmodulin through distinct domains. This calcium dependency gives DMI3 a straightforward role in the downstream transmission of the signal. Its substrate *MtIPD3* (for *Interacting Protein of DMI3*), homolog of CYCLOPS in *L. japonicus*, shows no similarity with proteins of known function, with the exception of its nuclear localisation signal (Messinese et al. 2007).

This quick glance to the localisation and function of the SYM proteins immediately highlights two central aspects: the identification of the nucleus as the main

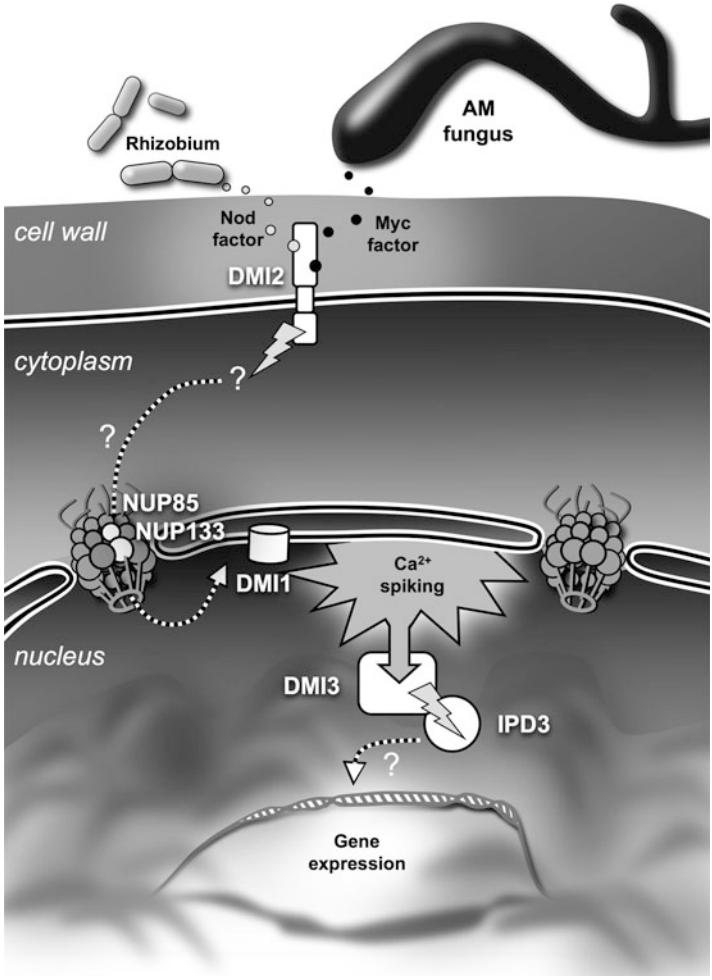

Fig. 2 The 'common' SYM pathway. The panel shows a schematic view of the proteins that are involved in the so-called SYM pathway, the plant signalling pathway that transduces both signals from nitrogen-fixing rhizobia (Nod factor) and AM fungi (Myc factor). The first known element of this common signalling pathway is the receptor-like kinase DMI2 from *M. truncatula* (homolog of SYMRK in *L. japonicus*). Its extracellular domain is thought to be involved in the recognition of both Nod and Myc factors, while the cytoplasmic domain shows a kinasic activity (lightning sign). The target of DMI2 is not known, but either this protein or a secondary messenger is supposed to eventually reach the nucleus. In fact two nucleoporins (NUP85 and NUP133) have been characterised in *L. japonicus*, whose functionality is required for downstream signalling. The nuclear envelope is also the site where DMI1, a putative cation channel homolog of *L. japonicus* CASTOR and POLLUX gene products, is localised. The nuclear envelope is a well-known calcium storage compartment, and the SYM pathway includes the generation of a calcium spiking signal in the nucleus and perinuclear cytoplasm. Although Ca^{2+} is not the preferential cargo of DMI1, a role for this channel has been proposed in the generation of the Ca^{2+} spiking (Charpentier et al. 2008). The calcium and calmodulin-dependent kinase DMI3 (CCaMK in *L. japonicus*) is localised in the nucleoplasm and is supposed to decode the calcium signal and phosphorylate the last known protein of the common SYM pathway, IPD3 (homolog of CYCLOPS in *L. japonicus*), whose function remains unknown, but localises upstream gene regulation. Please refer to Parniske (2008) for a compendium of the known SYM gene homologs in different AM host plants

target of the signalling pathway and the involvement of calcium as an intermediate messenger, indispensable for the activation of the nuclear kinase DMI3.

4.1 Calcium as a Secondary Messenger in AM Establishment

While the importance of intranuclear calcium signals was fully appreciated only after the demonstration of DMI3 nuclear localisation (Smit et al. 2005), the involvement of Ca^{2+} signals in AM perception has been postulated since the identification of a mycorrhizal phenotype in the *dmi3* mutants (Lévy et al. 2004). Sound support to this hypothesis came from the description of calcium spiking in the perinuclear area (Erhardt et al. 1996; Walker et al. 2000) and more recently in the nucleoplasm (Sieberer et al. 2009) of root hairs exposed to Nod factors. The first descriptions of calcium signals in response to AM fungal diffusible factors (Navazio et al. 2007; Kosuta et al. 2008; Chabaud et al. 2010), although somehow expected, represented a long awaited support to the theory of the common SYM pathway.

If, on one hand, the identification of a common signalling pathway controlling the establishment of two diverse symbioses in legumes has important ecological, developmental and evolutionary implications (Parniske 2008; Bonfante and Genre 2008; Markmann and Parniske 2009), on the other hand, it raises the problem of how legumes may distinguish between an AM fungus and a rhizobium and organise the appropriate accommodation of either one inside the root tissues, when both signals apparently converge over the same transduction pathway. Under this respect, stimulating clues come from AM-related calcium signals. Ca^{2+} is the most widespread intracellular messenger and transient elevations of its concentrations couple a wide array of extracellular stimuli to specific physiological responses (Hetherington and Brownlee 2004; Sanders et al. 2002). With so many signalling pathways based on the variation of Ca^{2+} concentration, eukaryotic cells have evolved sophisticated ways to generate and decode Ca^{2+} signals in a pathway-specific manner (the so-called *calcium signature*). Beside the specific subcellular localisation of Ca^{2+} release and the combined action of other intracellular messengers, plant cells can originate different calcium signatures in response to different stimuli by changing the duration of the Ca^{2+} concentration peaks as well as their frequency (Allen et al. 1999; Harper and Harmon 2005). AM-related nuclear calcium spikes have in fact been described as less frequent and more irregular compared to those induced by Nod factors (Sieberer et al. 2009; Chabaud et al. 2010) and recent hypotheses have been proposed, concerning the possible impact of such frequency modulations in decoding the two respective signals (Kosuta et al. 2008; Hazledine et al. 2009). If this hypothesis will further be confirmed, the question remains open concerning the mechanism by which the same Ca^{2+}-dependent enzyme, DMI3, can in turn distinguish the two frequency-coded messages.

Due to its positioning within the SYM pathway, calcium spiking can also be used as a reporter of the activation of this signal transduction pathway. This led, for

example, to the identification of AM-responsive districts in the root epidermis, localised between 1 and 2 cm from the tip of young lateral roots (Chabaud et al. 2010). Further studies are expected to clarify to what extent the calcium spiking response (and hence the SYM pathway) is also involved after cell entry, for fungal colonization of epidermal and cortical cells. Under this respect, the observation of intense nuclear calcium spiking in the small group of epidermal cells immediately surrounding each AM hyphopodium (Chabaud et al. 2010) confirmed that the response is directly related to the colonization process and that the SYM pathway is active in epidermal cells. This was previously only suggested by the phenotype of SYM gene mutants where fungal growth was arrested within the epidermal layer (Kistner et al. 2005; Novero et al. 2002) and gene expression was altered in the same cells (Chabaud et al. 2002).

5 Fungal Accommodation

Upon the development of a hyphopodium on the outer wall of root epidermal cells both the plant and fungus enter a novel developmental program that culminates in fungal accommodation inside the root cell lumen and symbiosis full functionality. Among the epidermal cells where the nuclear calcium spiking is observed, a few of those in direct contact with the hyphopodium dramatically reorganise their cytoplasm. The first visible sign of this response is the migration of the nucleus at the fungus contact site (Genre et al. 2005). Interestingly, such repositioned nuclei show the highest spiking frequencies (Chabaud et al. 2010), suggesting that the initiation of the accommodation program correlates with a strong activation of the SYM pathway – whose nucleus-oriented directionality has been discussed above. During the following few hours, the repositioned nucleus is progressively surrounded by an aggregation of cytoplasm, which expands into a columnar shape as the nucleus moves again from the contact site to the opposite side of the cell. Such a broad and roughly linear cytoplasmic aggregation is called the prepenetration apparatus (or PPA; Genre et al. 2005), as it predicts the intracellular path where the penetration hypha will grow. The PPA includes cytoskeletal fibres as well as the whole exocytotic apparatus, from a large accumulation of endoplasmic reticulum to numerous Golgi stacks, trans-golgi network and secretory vesicles (Genre et al. 2005, 2008). Remarkably, PPA assembly requires 6–7 h after hyphopodium adhesion. During this time, hyphopodium development appears to be totally arrested, which strongly suggests that fungal growth, at least at this stage, is controlled by the plant process that prepares cell entry.

The direct cell-to-cell contact may favour an intense and rapid signal exchange, which could include – but not be limited to – diffusible compounds such as the Myc factor. Based on the comparison with plant pathogenic interactions, the release of effector molecules from the hyphopodium seems likely (Catanzariti et al. 2006; O'Connell and Panstruga 2006; Valent and Khang 2010). Indeed, the observed interpenetration of fungal and plant cell walls at the contact site (Bonfante and

Genre 2010) suggests an intense local remodelling of the apoplast, which is compatible with protein secretion and bioactive molecules release (O'Connell and Panstruga 2006).

On the plant side, little information is available concerning secreted molecules that may be active during the initiation of the symbiotic phase. Most of the host plant known mutants showing a block to epidermal cell penetration by the fungus bear mutations in one of the SYM gene homologs (Parniske 2008). Nevertheless, the screening of mutagenised lines from tomato, maize and *Petunia* has led to the identification of a few mutants whose phenotype involves the inhibition of fungal proliferation, including abnormal hyphopodium development (Barker et al. 1998; David-Schwartz et al. 2001; Paszkowski et al. 2006; Reddy et al. 2007). At least in tomato, such effects are cancelled by the presence of WT plants in the vicinity of the mutants, strongly suggesting that the inhibition of fungal proliferation is due to the lack of so-far unidentified root secreted molecules (Cavagnaro et al. 2004).

The occurrence of signalling events during AM colonisation of the inner root tissues is suggested by the observation of long-distance responses in the plant cells (Fig. 3). As soon as the PPA is being completed in an epidermal cell, nuclear repositioning in front of its terminal site is reported in the underlying cortical cell, associated with the initiation of a new PPA (Genre et al. 2008). This implies that the site where the fungus will cross the epidermal/cortical cell wall is being marked before the fungus has even started penetrating the epidermal cell. Analogous and even more striking clues for the involvement of long-range signals are observed in the inner cortex of Paris-type mycorrhizas, such as those that develop in carrot. Here, fungal colonisation of the root tissue proceeds from cell to cell. PPAs in progressively advanced stages of development are observed in several cells ahead of the developing hyphal tip, indicating that a signal triggering the accommodation process is translocated on a relatively long distance (Genre et al. 2008). Lastly, plant cell responses in the non-colonised cells adjacent to arbuscules have been detected with both cellular and molecular methods. Microtubule rearrangement in the vicinity of arbusculated cells of *M. truncatula* (Blancaflor et al. 2001) and gene regulation in non-colonized cortical cells from the colonised areas of *M. truncatula* (Harrison 2005) and tomato roots (Balestrini et al. 2007) have both been interpreted as the result of cell-to-cell signal transfer. Interestingly, lysophosphatidylcholine extracted from arbuscule-containing roots has been shown to induce the upregulation of AM-specific phosphate transporters in non-colonised roots, suggesting that this molecule could be one of such cell-to-cell signals (Drissner et al. 2007).

Intense membrane dynamics occur within the PPA, as documented by the rapid uptake of the endocytotic marker FM4-64 (Genre et al. 2005) and the simultaneous presence of exocytotic vesicles and endosomes (Genre et al. 2008). Endosome-mediated signalling is a well-known mechanism, which, especially in plant cells, grants the rapid translocation of active receptors from the perception site on the plasma membrane to the cellular domain where the response is started (Geldner and Robatzek 2008). From this point of view one can reasonably speculate that the PPA acts as a route for signalling endosomes, thanks to the dynamism of its vesicular

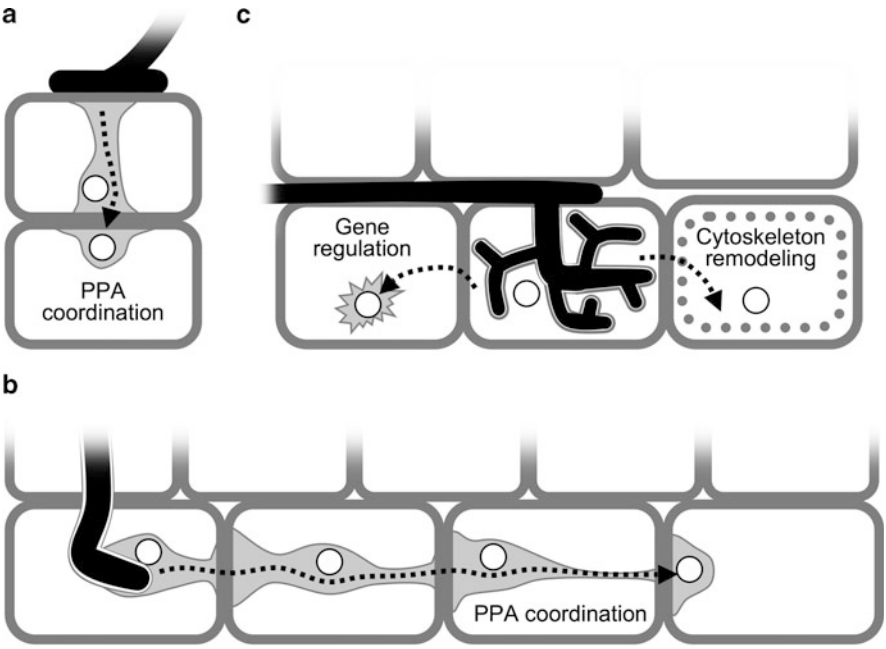

Fig. 3 Long-range signal transmission during AM colonisation of the root tissues. Several cellular clues indicate the existence of diffusible signals that induce prepenetration responses in the inner root tissues before the cells are in direct contact with the fungus. The diagram in (**a**) shows the initiation of a PPA in the outer cortical cell, in line with the fully assembled PPA of the overlaying epidermal cell. The diagram in (**b**) shows a similar but more extensive coordination in PPA assembly within a group of inner cortical cells. Prepenetration responses appear to be progressively less advanced with increasing distance from the growing hyphal tip, but PPA alignment along the cell file is evident. The diagram in (**c**) summarises the observation of arbuscule-specific gene regulation and cytoskeleton remodelling in non-colonised cells adjacent to the arbusculated ones. Both are suggestive of a signal diffusion from the arbuscule to activate the neighbouring cells in preparation for potential fungal entry

content. The longitudinal orientation of microtubules and microfilaments inside the PPA aggregate (Genre et al. 2005) also supports this view. This would explain how prepenetration responses coordinate between adjacent cells, but does not clarify whether the upstream stimulus has a direct fungal origin or derives from a secondary plant signal. The location of such responses deep within the root cells and tissues currently hinders our attempts to solve this conundrum. Only the availability of genomic information and the application of genetic tools on the fungus will likely shed light on such aspects.

A remarkable trait stands out from the study of prepenetration responses in AM host plants: nuclear movement, cytoskeleton arrangement, focal concentration of exocytotic elements and the apparent directional translocation of signalling factors are suggestive of cell polarisation processes. The onset of polarity is common to many plant developmental processes, including responses to pathogens (Genre et al. 2009)

but also apical growth and hormonal fluxes. And indeed the directional transport of auxin is known to be involved in the establishment of cell polarity in a self-inducing mechanism (Lee and Cho 2006; Feraru and Friml 2008).

Nevertheless, a correlation between AM colonisation and hormone activity has so far only been highlighted on organ and systemic scale. Auxin levels increase in mycorrhizal root systems, but are unrelated to the distribution of colonised areas (Jentschel et al. 2007). Auxin-upregulated genes, such as the leghemoglobin *Lb29* from *Vicia faba*, were shown to be also induced during AM colonization, indirectly confirming the AM-induced increase in auxin levels (Ludwig-Müller and Güther 2007). The effect of AM on ethylene, abscisic acid and jasmonate-related compounds, as well as the expression of key genes in hormone biosynthetic pathways shows important differences depending on the fungal species (López-Ráez et al. 2010). Even more controversial is the involvement of jasmonic and salicylic acids in regulating AM-induced defense (Gutjahr and Paszkowski 2009; Hause and Schaarschmidt 2009). All these data indicate a role for hormone-based signals especially on the systemic effects of the AM interaction, which range from the long described "growth effect" observed in mycorrhizal plants (Smith and Read 2008) to the large-scale reprogramming of regulatory networks as it is being highlighted by transcriptomic analyses and microarray assays (Liu et al. 2007; Fiorilli et al. 2009). Furthermore, beside plant hormones, long distance signals might also include mobile microRNAs, as recently suggested by expression analyses (Gu et al. 2010; Branscheid et al. 2010).

6 Signalling During Symbiosis Functioning

Arbuscule appearance inside the lumen of inner cortical cells hallmarks the establishment of mutualism. The extensive periarbuscular interface and membrane in fact mediate the intense nutrient exchange that characterises AM symbiosis and represents the basis of its ecological and evolutionary success. Signalling mechanisms partly similar to those controlling intracellular fungal growth in the outer tissues are predicted to control arbuscule development also, as suggested by the reiteration of the PPA model prior to the development of new fungal branches (Genre et al. 2008). Nonetheless, the accommodation program within the inner cortex develops unique processes, which can be envisaged through both cellular and molecular investigations. The genetic control of arbuscule development is only partly ruled by the SYM pathway. Most SYM gene mutants either block fungal penetration in the outer cell layers or develop normal and functional arbuscules when such surface block is taken over (Morandi et al. 2005). Very few exceptions are known: a *castor* mutant (*LjSym4-1*), in *L. japonicus*, was shown to develop fewer arbuscules and more intercellular hyphae than the wild type, suggesting that the mutation affects cortical cell entry (Novero et al. 2002). A very similar phenotype derives from RNAi-mediated downregulation of the *Vapyrin* gene in *M. truncatula*, but the gene does not belong to the SYM group (Pumplin et al.

2010). Interestingly, the *Vapyrin* gene is unique to the plant kingdom but absent in the AM non-host *Arabidopsis*, suggesting a possible AM-specific – although unknown – function for the encoded ankyrin-like protein.

Morphological evidence for the diversity of the cell responses in inner cortical versus epidermal and outer cortical cells include the overall morphology of the PPAs (thinner and roughly linear in the outer tissues, broader and bulged in the inner cortex), the final positioning of the nucleus (which persists in the cell centre during the whole arbuscule life span), the decondensation of chromatin, only observed in the inner cortex and suggestive of more intense transcriptional activity (Genre et al. 2008). How all these processes are controlled and specifically triggered in the cells that will contain arbuscules remains to be clarified.

When arbuscules reach maturity, filling up most of the cell volume, the nutrients they transfer also seem to have a role as signals. Javot et al. (2007) have elegantly demonstrated that if the plant does not receive P from the fungus – due to either RNAi silencing or mutation of the arbuscule-specific *MtPT4* phosphate transporter – cortical cell colonisation is followed by an anticipated process of fungal senescence, resulting in small arbuscules made of thick, short branches. Such arbuscules septate and die much earlier (3–4 days after inoculation) than normal arbuscules developed in wild-type plants (with a reported life span of over 8 days). These observations indicate that phosphate, the most important nutrient provided to host plants by AM fungi, can act upstream a signalling pathway that controls arbuscule morphogenesis and senescence. Although this result was obtained by knocking down a plant transporter, it strongly suggests that the fungal efficiency in P delivery has a major importance for symbiosis establishment.

Altogether, the occurrence of signal exchanges inside the arbusculated cells is more than just likely. A strict coordination between the two partners is required during the whole development of the interaction, and the complexity of arbuscule differentiation and functioning makes these structures the site where plant–fungus signalling must inevitably reach the highest intensity. Among such signals, the very nutrients that the plant and the fungus exchange through the periarbuscular interface are to be considered as an important and perhaps major component. It is expected that over the next few years AM-related research, currently focussed on the presymbiotic signals, will concentrate on the signalling events that associate with arbuscule development, function and senescence.

7 Conclusions

AM symbiosis is the result of a delicate balance, where both partners release signals, respond to each other and coordinate their developmental programs, leading to the accommodation of a eukaryote inside another eukaryote. This rather uncommon event presents unique peculiarities in the case of AM. The hosted organism, in fact, as a true endosymbiont, develops inside the plant cells; nevertheless, its intracellular structures maintain direct communication with the extraradical

mycelium that explores the soil. The absence of septa and the strong cytoplasmic streams that characterise AM fungi lead to a condition where a true cellular continuity can be envisaged within the whole organism.

Compared to such a complex biological system, our current view of the cellular and molecular mechanisms that rule AM is not simply limited, but often biased. First, a substantial lack of information concerning the fungus impacts both our knowledge and the overall picture that we get of AM interactions. Besides leaving many fundamental aspects of AM fungal biology completely obscure, such a 'green shift' is responsible for the common idea that AM host plants hold major control over the symbiosis. This is supported by many experiments, where the mutation of a single plant gene results in the block of fungal development at the hyphopodium stage. Nevertheless, the fact that fungal mutants have not so far been obtained does not exclude that fungal control is just as strong. Similarly, plant genetic manipulation has allowed detailed investigations of the cell responses during fungal colonisation, but we know very little about the morphogenetical programs that originate all of the symbiotic structures of AM fungi, from hyphopodia to intracellular hyphae, coils, vesicles and arbuscules. Once again, the lack of appropriate tools strongly affects our possibilities to investigate these aspects. The achievement of transient genetic transformation in *Glomus intraradices* (Helber and Requena 2008) envisages the dawn of a new era in AM research, where this methodological gap can be filled.

A somehow similar bias can be ascribed to the appeal of simplification. The advancements that have been made on the identification of the SYM signalling pathway, far from providing a complete signal transduction sequence, have polarised the attention of researchers on this one signalling mechanism. It should anyway be noticed that an increasing set of data demonstrate the existence of signal transduction processes in AM that transcend the SYM pathway. Recent examples include the regulation of AM-specific marker genes in SYM mutants (Gutjahr et al. 2008; Kuhn et al. 2010). Besides being still partially obscure, the presymbiotic signalling in AM may thus be significantly more complex than expected and research in the next years is likely to provide us with both answers and new questions.

Altogether, the signals and responses that we know to be controlling AM establishment fall into well-known mechanisms of all eukaryotic cells. Signal transduction through elicitor perception, receptor activation, calcium-mediated signals, phosphorylation cascades; cell responses involving cytoplasm aggregation and focal exocytosis; and the reprogramming of metabolic and physiological pathways on a local and systemic scale. All of these events are common to most plant (and animal) interactions. A challenge for the near future will be to understand how the same basic mechanisms are routed towards one conclusion or another. For example, cell responses that involve nuclear repositioning, cytoplasmic aggregation, cytoskeletal remodelling and local membrane dynamics are described in both pathogenic and symbiotic interactions, but the same cellular machinery is obviously able to organise defense responses, such as the development of cell wall appositions underneath the penetration peg of a pathogen, or accommodation processes, such as

the assembly of the perifungal interface to host an AM fungus. Molecular mechanisms such as the regulation of defense-related gene expression are also known to be involved in both mutualistic and pathogenic interactions (Paszkowski 2006b), but the control, modulation and reprogramming of such responses must depend on specific signals and signalling cascades that identify each interacting microorganism.

If, on one hand, presymbiotic signals have been one of the most difficult conundrums that AM research has faced so far, on the other hand, they take place during the most accessible phase of AM development. The plant and fungus are still separated and can be manipulated independently to highlight the effects of diffusible molecules and study the details of cellular and molecular responses. But starting from hyphopodium adhesion, the tools we can apply to address the investigation of signalling events inside the root tissues are indeed less powerful. Many questions remain therefore open. For example, experimental evidence shows that the PPA predicts intracellular fungal growth, but how is the penetrating hyphal tip induced to exactly follow that membranous track, rather than break into the plant cytoplasm? Similarly, the prepenetration responses that develop in the cortical cells do not extend indefinitely. Each infection event leads to the colonisation of a limited zone of the root, but how such limits are set and how fungal colonisation of the cortex is controlled by either the plant or the fungus remains largely unexplored. Such examples strongly suggest the existence of a complex network of signalling mechanisms throughout the AM symbiotic cycle, and these clues will likely be the germ of AM signal research for the next years.

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Signalling and Communication in the Actinorhizal Symbiosis

Claudine Franche and Didier Bogusz

Abstract More than 200 species of non-legume dicotyledonous plants, mostly trees and shrubs, belonging to eight different families and 24 genera can enter actinorhizal symbioses with the nitrogen-fixing actinomycete *Frankia*. Actinorhizal nodules consist of multiple lobes, each of which displays a lateral root structure with infected cells in the expanded cortex. Whereas the key molecules involved in the molecular dialogue between the symbiotic partners have not yet been characterized, the development of genomic and molecular tools both in *Frankia* and in some actinorhizal plants has contributed to a better understanding of this original endosymbiosis.

1 Introduction

Actinorhizal plants are a highly diverse group of angiosperms capable of forming nitrogen-fixing root nodules with the soil actinobacteria *Frankia* (Huss-Danell 1997; Wall 2000). Phylogenetically, they occur among 25 genera distributed in eight plant families and four orders with wide-ranging global distributions (Table 1). Representative species and genera populate temperate forests (*Alnus*, *Comptonia*, *Myrica*), coastal dunes (*Casuarina*, *Myrica*, *Hippophae*), alpine biotopes (*Alnus*, *Dryas*) and dry areas (*Ceanothus*, *Purshia*, *Cercocarpus*) (Silvester 1976). Actinorhizal plants share common features; with the exception of *Datisca* which has herbaceous shoots, they are perennial dicots and include woody shrubs and trees. Most actinorhizal plants are capable of high rates of nitrogen fixation comparable to those found in legumes, and they can sustain mycorrhizal associations as well (Torrey and Tjepkema 1979; Dawson 2008).

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Table 1 Classification of actinorhizal species and frequency of nodulation within families (adapted from Dawson 2008)

Plant orders	Family	Nodulated/total genera	Genus	Number of species
Fagales	*Betulaceae*	1/6	*Alnus*	47
	Casuarinaceae	4/4	*Allocasuarina*	54
			Casuarina	16
			Ceuthostoma	2
			Gymnostoma	18
	Myricaceae	2/3	*Comptonia*	1
			Myrica	28
Rosales	*Eleagnaceae*	3/3	*Eleagnus*	38
			Hippophae	2
			Shepherdia	2
	Rhamnaceae	8/55	*Adolphia*	2
			Ceanothus	31
			Colletia	4
			Discaria	5
			Kentrothamnus	1
			Retanilla	2
			Talguenea	1
			Trevoa	2
	Rosaceae	5/100	*Cercocarpus*	4
			Chamaebatia	1
			Cowania	1
			Dryas	1
			Purshia	2
Cucurbitales	*Coriariaceae*	1/1	*Coriaria*	5
	Datiscaceae	1/3	*Datisca*	2

Due to these symbiotic properties, actinorhizal plants contribute to improve soil fertility in disturbed sites and grow well under a range of environmental stresses such as high salinity, heavy metal pollution and extreme pH. This facility for adaptation is responsible for the great interest in actinorhizal plants, particularly in several species of *Casuarinaceae* such as *Casuarina glauca* and *C. equisetifolia*, which are widely planted in the tropics and subtropics to stabilize sand dunes, produce fuel wood, rehabilitate area with industrial waste and to increase soil fertility in agroforestry systems (Dawson 1986; Diem and Dommergues 1990; Diouf et al. 2008; Zhong et al. 2010).

Our basic knowledge of the symbiotic association between *Frankia* and actinorhizal plants is still poorly understood, even though it offers striking differences with the *Rhizobium*–legume symbiosis (Pawlowski and Bisseling 1996; Wall 2000; Vessey et al. 2005; Laplaze et al. 2008; Pawlowski and Sprent 2008). *Frankia* is a filamentous, branching, Gram-positive actinomycete, whereas Rhizobia are Gram-negative unicellular bacteria. Whereas Rhizobia only enter symbiosis with plants from the legume family and with one non-legume, *Parasponia*, *Frankia* can interact with a diverse group of non-legume

dicotyledonous plants. At the structural level, legume root nodules have peripheral vascular bundles and infected cells in the central tissue, whereas actinorhizal nodules display a lateral root structure with a central vascular bundle and peripheral infected cortical tissue (Duhoux et al. 1996).

The molecular understanding of regulatory events in actinorhizal nodulation is limited by several factors (Laplaze et al. 2008). One of the major limitations is the lack of genetic approaches for the host actinorhizal plants that are trees and shrubs. Additionally, because of their slow growth and high phenolic content, woody plants such as actinorhizal trees are generally less amenable to physiological and molecular analyses than herbaceous plants. Concerning the microsymbiont *Frankia*, the main obstacles are the difficulty to obtain pure culture of the actinobacteria, the slow-growth rate and the filamentous structure of *Frankia* which makes it more difficult to culture in either liquid or solid medium, and the lack of genetic and mutagenesis tools (Lavire and Cournoyer 2003).

Recently, the development of genomics both in *Frankia* (Normand et al. 2007a) and in some actinorhizal plants such as *C. glauca* (Hocher et al. 2006, 2011), together with the possibility to obtain transgenic actinorhizal plants following *Agrobacterium* gene transfer (Diouf et al. 1995; Franche et al. 1997; Gherbi et al. 2008b; Svistoonoff et al. 2010a), offer new approaches to understand the molecular basis of the actinorhizal process. In this review, we will highlight recent progress in the molecular knowledge of the early stages of the actinorhizal symbiosis and recent data concerning the identification of genes involved in the *Frankia* factor (s) signalling pathway. We mainly focus on the interaction between *Frankia* and the tropical tree *C. glauca*, which is one of the most advanced actinorhizal models.

2 The Microorganism *Frankia*

2.1 General Features of Frankia

The symbiotic microorganism *Frankia* is the only member of the family *Frankiaceae* in the order *Actinomycetales* (Normand et al. 1996). *Frankia* is not an obligate endosymbiont and can occupy two distinct ecological niches, the root nodule and the soil. Besides its ability to develop a symbiotic interaction with roots, *Frankia* is a pleiomorphic bacterium that develops specific structures, vesicules and spores, that are critical for its survival in the rhizosphere (Newcomb and Wood 1987; Benson and Silvester 1993). Spores are formed in sporangia at the mycelial tips and are thought to contribute to the dissemination and survival of the microorganism. Vesicules are specialized nitrogen-fixing cells that develop at the tips of growing vegetative hyphae when exposed to nitrogen starvation and oxygen. They are surrounded by a laminated lipid envelope containing a mixture of hopanoid lipids which are pentacyclic bacteriohopanes. The thickness of the vesicle envelope increases with an increase in O_2 and helps to maintain partial oxygen pressure at

levels that are not labile for the dinitrogenase complex (Parsons et al. 1987; Berry et al. 1993).

2.2 *Specificity of the Symbiotic Association*

Since the first report of an infective *Frankia* strain from nodules of *Comptonia peregrina* in 1978 by Callaham et al., hundreds of *Frankia* isolates have been described. Individual strains can nodulate actinorhizal plants from different orders, thus implying that the host origin is not always a determining characteristic for strain classification. On the basis of cross-infectivity studies and genotypic and phylogenetic approaches, three cohesive *Frankia* groups with distinct host ranges have been defined, as well as a fourth large group of "atypical" strains (Benson and Clawson 2000; Clawson et al. 2004; Hahn 2008; Normand and Fernandez 2009). *Frankia* from Group I include strains which nodulate members of the hamamelid families *Betulaceae*, *Casuarinaceae* and *Myricaceae*. *Frankia* from Group II are typically associated with members of the *Coriariaceae*, *Datiscaceae*, *Rosaceae* and *Ceanothus* of the *Rhamnaceae*. Group II is characterized by low diversity, supporting the hypothesis of a recent origin for symbiosis in this lineage. Group III is still poorly known; strains appear to nodulate most *Ceanothus* sp. and also appear in *Myricaceae*, *Eleagnaceae*, *Rosaceae*, *Betulaceae* and *Gymnostoma* of the *Casuarinaceae* family.

Based on their different requirements with respect to *Frankia* strains, actinorhizal plants have been divided into promiscuous and non-promiscuous species (Valdes 2008). Non-promiscuous plant genera include *Allocasuarina*, *Casuarina*, *Eleagnus* and *Hippohae*, whereas in greenhouse conditions, *Myrica* can be nodulated by most *Frankia* strains. Although specific, the genera *Alnus* and *Gymnostoma* can sometimes be nodulated by *Frankia* strains from the *Eleagnaceae* infective group. These data suggest that the plant–actinobacterium interaction could be less specific than the legume–Rhizobium symbiosis.

2.3 *Molecular Tools and Genomics of* Frankia

The development of genetic tools for *Frankia* is progressing slowly and remains a major hurdle for studying the molecular dialogue between the symbiont and the host plant (Lavire and Cournoyer 2003; Franche et al. 2009). Although there have been several attempts to develop shuttle vectors from native *Frankia* plasmids, there is still no known reliable gene transfer system for the actinomycete (John et al. 2001; Lavire et al. 2001; Xu et al. 2002). Recently, potential transformants of the strain CcI3 were reported following electroporation with a vector carrying a tetracyclin resistance gene with a codon usage similar to the one of *Frankia*, and driven by a native promoter. Antibiotic selection in liquid culture enabled the

growth of tetracyclin-resistant cells (Kucho et al. 2009). However, analysis of the genomic DNA from the putative transformants revealed that the marker gene was not integrated in the host. An alternative approach based on the genetic transformation of a uracil-requiring pyrF mutant of CcI3 with a vector carrying the wild-type *pyrF* gene is in progress (Kucho et al. unpublished data).

To overcome this limitation and to gain new insights into the nitrogen-fixing actinobacteria, the genomes of three *Frankia* strains including CcI3, ACN14a and EAN1pec, which respectively nodulate *C. glauca*, *Alnus glutinosa* and *Eleagnus angustifolia*, were compared (Normand et al. 2007a, b; Rawnsley and Tisa 2007). These genomes were found to be circular and to exhibit high G + C content. The most striking feature among the three genomes was their sizes, which ranged from 5.43 Mbp for the narrow host range strain CcI3 symbiotic of *Casuarina*, to 9.04 for the broad host range strain of *Eleagnus* EAN1pec. Besides, no significant symbiotic island or homologues of the common *nodABC* genes from Rhizobium, which are required for the synthesis of the core structure of the Nod factors, were identified in the genomes.

The availability of *Frankia* genomes has paved the way for the use of bioinformatic approaches such as proteomics. A proteome approach was used on *F. alni* to study the physiology of *Frankia* and to analyse the biochemical events associated with the switch from N_2-replete to N_2-fixing conditions (Alloisio et al. 2007). Up-regulation of stress proteins and proteins involved in N assimilation was observed under nitrogen starvation. Additional studies were performed on strain CcI3 to compare proteome from free-living and symbiotic cells (Mastronunzio et al. 2009). Solute-binding proteins were the most common class of secreted proteins observed in the symbiotic proteome, whereas hydrolytic enzymes were rarely detected. These data suggest that plant cell wall and/or membrane digestion is not likely to be used by the actinobacteria during the colonization process.

Results of a transcriptome analysis were recently published in the *F. alni* ACN14a strain following the expression profiling of 6,607 genes in free-living nitrogen conditions and symbiotic state (Alloisio et al. 2010). This enabled identification of a large number of up-regulated symbiotic genes such as genes related to nitrogen fixation, transcriptional regulation, signalling processes, protein drug export, protein secretion, liposaccharide, and peptidoglycan biosynthesis. Since strain ACN14a can establish a symbiotic relationship with plants of different actinorhizal families, the impact of the host plant on the transcriptome of the symbiont was then studied. Microarray analyses of the transcriptomes in ACN14a from *A. glutinosa*, *Alnus nepalensis*, *Myrica gale* and *Morella rubra* did not reveal any significant differences.

Six more genomes of *Frankia* are currently being sequenced, and it is expected that the comparative analysis of these additional genomes with different features and host specificities will enable a deeper understanding of *Frankia* in free-living conditions as well as during different stages of the interaction with hosts of phylogenetically distant plant families.

3 The Actinorhizal Nodule

3.1 The Infection Process

Frankia can penetrate the plant root either intracellularly or intercellularly, depending on the host plant (Fig. 1). In "higher" *Hamamelidae* such as *Alnus*, *Myrica*, *Comptonia*, *Casuarina* and *Gymnostoma*, the infection process proceeds through root hair deformation and penetration. The process starts with root hair curling following the exchange of unknown signals between *Frankia* and the host plant (Prin and Rougier 1987). After invagination of growing filaments of *Frankia* into the curled root hairs, infection proceeds intracellularly in the root cortex (Callaham et al. 1979; Berry et al. 1986). *Frankia* hyphae are surrounded by a plant-derived encapsulation layer enriched in polygalacturonans, in addition to host plasmalemma and cytoplasm. This interfacial matrix is the equivalent of the infection thread wall in legume nodules (Berg 1990, 1999). Upon infection, cell divisions occur in the root cortex near the infected hairs, leading to a small external protuberance called a prenodule, which consists of *Frankia*-infected and uninfected

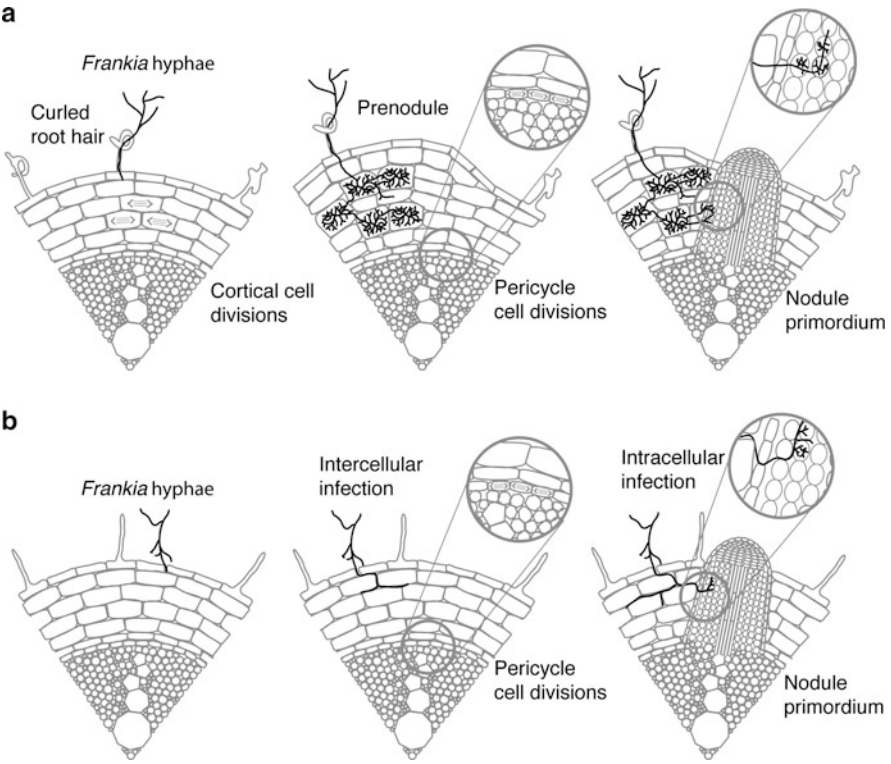

Fig. 1 Stages of root infection by *Frankia*. (**a**) Intracellular infection; (**b**) Intercellular infection

cells (Fig. 2a, b) (Berry and Sunnel 1990). Since the formation of the nodule primordium does not involve prenodule cells, it is assumed that the prenodule could be a primitive symbiotic organ (Laplaze et al 2000a). The nodule primordium results from mitotic activity in pericycle cells located opposite a protoxylem pole close to the prenodule (Callaham and Torrey 1977; Duhoux et al. 1996). The infection of the developing nodule appears to be coordinated with the expansion of post-meristematic cells.

An intercolonization process is observed in 17 genera distributed throughout the families *Eleagnaceae*, *Rhamnaceae*, *Rosaceae*, *Datiscaceae* and *Coriariaceae*, thus representing 70% of the total actinorhizal species. In this infection pathway, root hairs are neither deformed nor invaded by the actinobacteria. *Frankia* enters the root by growing through the intercellular spaces between adjacent epidermal cells, penetrating the middle lamella, and progresses apoplastically through cortical cells within an electron-dense matrix secreted into the intercellular spaces (Miller and Baker 1985; Liu and Berry 1991; Valverde and Wall 1999; Wall and Berry 2008). Once the nodule primordium has developed from the pericycle, intracellular penetration by *Frankia* and the formation of infection threads is initiated acropetally in developing cortical cells of the nodule lobe primordium. In this infection process, the prenodule stage is not observed. Although the infection process has been studied in relatively few plant species, it is suggested that the ancestral mode of infection is via intercellular penetration (Swensen and Benson 2008).

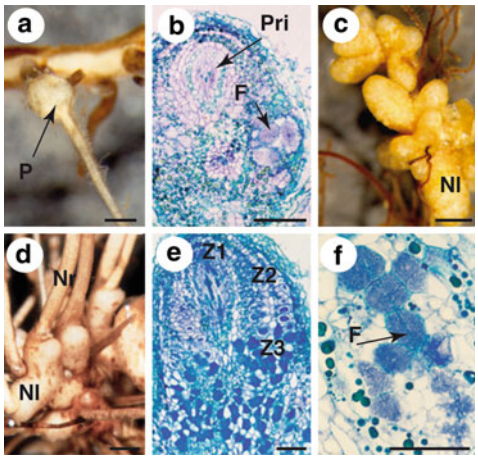

Fig. 2 Actinorhizal nodulation in Casuarina. (**a**) Prenodule (P) resulting from the infection of *C. glauca* by *Frankia* CcI3. Bar = 1 mm. (**b**) Longitudinal section of a prenodule showing the large cortical cells infected by *Frankia* (F) and the emerging nodular primordium (Pri). Bar = 100 µm. (**c**) Three-month-old nodules with multiple nodular lobes (Nl) on the root system of *Allocasuarina verticillata*. Bar = 1 mm. (**d**) Three-month-old nodule with nodular lobes (Nl) and nodular roots (Nr) on the root system of *C. glauca*. (**e**) Longitudinal section of an actinorhizal nodule lobe showing the meristematic zone (Z1), the infection zone (Z2) and the fixation zone (Z3). Bar = 100 µm. F: Enlargement and lignification of nodule cortical cells resulting from the infection by *Frankia* in *C. glauca*. Bar = 100 µm

3.2 Nodule Development

Actinorhizal nodules, referred to as actinorhiza, consist of multiple lobes, each of which resembles a modified lateral root without a cap (Fig. 2c). Nodule lobes have a central vascular bundle, a superficial periderm and both infected and non-infected cortical cells. The non-infected cells are thought to play an important role in the metabolism and transport of the primary product of N_2 fixation and assimilation. In the genera *Casuarina*, *Datisca*, *Myrica*, *Comptonia* and *Gymnostoma*, a so-called root nodule is also observed at the apex of each lobe (Fig. 2d) (Duhoux et al. 1996). This root nodule lacks root hairs, has a reduced root cap and displays negative geotropism. It might be involved in the diffusion of gas, especially oxygen, within and outside the nodule lobe.

Due to activity of the apical meristem, actinorhizal nodule lobes show an indeterminate growth pattern and have a developmental zonation in which specific patterns of gene expression are observed (Fig. 2e) (Duhoux et al. 1996). In zone 1, the apical meristem is free of *Frankia*. Adjacent to the meristem is an infection zone where some of the young cortical cells resulting from the meristem activity are infected by *Frankia*. The subsequent fixation zone contains both infected and uninfected cortical cells. Hypertrophied-infected cells are filled with *Frankia* hyphae that differentiate N_2 fixing vesicles. In some species like *Casuarina*, infected cells have a lignified cell wall and no vesicles are observed (Fig. 2f) (Berg and McDowell 1987). Finally, a basal senescence zone is observed in old nodules; plant cells and bacteria degenerate and nitrogen fixation is switched off. Although all nodule lobes have an apical meristem, the growth of individual lobes is limited. Additional branch lobes are formed as lateral primordia in the pericycle of the preceding lobe. In plantations of *Casuarina*, nodules of an average size of 25 cm have been observed.

4 Early Signal Molecules

4.1 Plant Signals

Actinorhizal nodule development only occurs under conditions of nitrogen deprivation and it is assumed that plant roots emit signals of still unknown nature that are perceived by *Frankia*. Root phenylpropanoids are prime candidates for these signalling molecules, since they govern plant-microorganism cross-talk in the rhizosphere and regulate the expression of *nod* genes in *Rhizobium* (Bhattacharya et al. 2010). In actinorhizal symbioses, the nodulation of *Alnus* sp. has been reported to be enhanced by the addition of flavonone (Benoit and Berry 1997) or flavonol (Hughes et al. 1999) compounds. There is also some evidence of chemoattraction and proliferation of *Frankia* in the rhizosphere of *Betula pendula*, but the chemical structure of the exuded substances remains unknown (Smolander and

Sarsa 1990). In the *Myricaceae* species, *Myrica gale*, seed phenolic extracts were shown to induce general reorganization of protein biosynthesis in several *Frankia* strains. Major trends include the up-regulation of oxidative stress proteins such as FeSOD, which could be involved in protection against host–plant defence (Bagnarol et al. 2007). In addition, the use of a transcriptomic approach to study the early stages of the infection process of *C. glauca* by *Frankia* revealed that several genes linked to the flavonoid biosynthesis pathway are up-regulated (Auguy et al. 2011). The role of flavonoids in the symbiotic process is currently under study following the down-regulation of the chalcone synthase gene using an RNA interference approach (Gherbi et al. 2008b) in *C. glauca*.

4.2 Frankia *Signals*

In both legumes and actinorhizal plants, the first observable event of the plant-microorganism dialogue is the curling of the root hairs (Callaham et al. 1979; Van Ghelue et al. 1997). Preliminary characterization of *Frankia* deforming factor(s) suggests that they are structurally divergent from the Nod factors of Rhizobia (Cérémonie et al 1999). Whereas in legumes Nod factors are heat-stable, amphiphilic and chitinase sensitive (Dénarié et al. 1996), *Frankia* factors from the symbiotic strain ACoN24d of *Alnus* were found to be heat-stable, hydrophilic, and resistant to endochitinase and exochitinase from *Streptomyces griseus*. Moreover, neither the broad host range *Rhizobium* sp. NGR234 nor its purified factors elicited root hair deformation in *Alnus* and *Casuarina*, thus suggesting that actinorhizal plants do not recognize lipo-chito-oligosaccharides. However, *N*-acetyl-glucosamine, the subunit of the Nod factor backbone, has been detected in the *Alnus* root hair deforming fraction, leaving open the possible existence of a Nod-factor-related *Frankia* compound (Cérémonie et al. 1999). The lack of a sensitive and reproducible bioassay to purify *Frankia* actinorhizal factor(s) remains one of the major obstacles for purifying the signalling molecules (McEwan et al. 1992).

4.3 Mitotic Activity in Infected Roots

Nod factors have been shown to induce mitotic activity in legume roots (Savouré et al. 1997). Using *Frankia* supernatant, no stimulation of mitosis on *A. glutinosa* roots was detected (Cérémonie et al. 1999). However, interesting data were obtained when the cell cycle promoter P*cdc2aAt* from *Arabidopsis thaliana* (Hemerly et al. 1993) was transferred to Casuarina. This gene encodes the P34cdc2 kinase, which is a key component in the regulation of the cell cycle, acting in the regulation of the G1 to S and G2 to M transitions (Hemerly et al. 1992; Wang et al. 2004). In non-inoculated roots of the *Casuarinaceae* tree *Allocasuarina verticillata* genetically transformed with the P*cdc2aAt-GUS* construct, the reporter gene expression was mainly observed

in primary and secondary meristems (Sy et al. 2007). Histological analysis of β-glucuronidase activity in *cdc2aAt-GUS* roots, inoculated for 7 days with *Frankia*, revealed that cells from the pericycle located opposite the protoxylem poles were deeply stained. These data suggest that upon *Frankia* infection, cells from the lateral roots, and notably the pericycle cells that give rise to nodule primordium, prepare to re-enter the cell cycle (Sy et al. 2007). It was also observed that adding *Frankia* supernatant to *cdc2aAt-GUS* roots for 2 days contributed to the induction of the *GUS* gene, indicating that molecules emitted by *Frankia* probably stimulate the cell cycle during the early stages of the interaction, like Nod factors in legumes. However, the molecules involved in this plant response remain to be characterized.

5 Signal Transduction Pathway

5.1 *Functional Characterization of the Candidate Gene* SymRK

The development of genetic and genomic tools for the model legumes *M. truncatula* and *Lotus japonicus* has greatly facilitated the cloning of genes required for root symbiosis (Geurts et al. 2005; Stacey et al. 2006; Oldroyd and Downie 2008; Madsen et al. 2010). Some of these genes were found to be involved in the establishment of both rhizobia and mycorrhiza symbioses, and designated as common Sym genes constituting the common Sym pathway. They include genes encoding a leucine-rich-repeat (LRR) receptor kinase (SymRK), cation channels, nuclear pore complex proteins, a calcium and calmodulin-dependent protein kinase (CCaMK) and a nuclear-coiled protein. The question was raised whether some of these symbiotic genes were shared in the signal transduction pathway in response to *Frankia* factors and rhizobial Nod factors.

To answer this question, a functional study of *CgSymRK*, a gene isolated from *C. glauca*, orthologous to the receptor-like kinase gene *SymRK* required for nodulation and mycorrhization in legumes, was undertaken (Endre et al. 2002; Stracke et al. 2002; Capoen et al. 2005). In legumes, this gene (also referred to as *DMI2* in *M. truncatula*) encodes a leucine-rich-receptor kinase that is needed for bacterial and endomycorrhizal infection, and Nod-factor-induced calcium spiking in the root epidermis. Down-regulation of *CgSymRK* resulting from an RNA interference approach revealed that the frequency of nodulated RNAi-*CgSymRK* plants was reduced twofold compared to control *C. glauca* plants (Gherbi et al. 2008a). In addition, a range of morphological alterations was observed in the down-regulated *CgSymRK* nodules. Whereas mature nodules in the control plants were multilobed, RNAi nodules were dramatically reduced in size and mostly consisted in a single thin lobe that did not fix nitrogen. Cytological analysis further showed that these aberrant nodular structures accumulated high levels of phenolic compounds and contained cortical-infected cells that were smaller than untransformed nodular lobes in controls. Additional experiments revealed that CgSymRK was also

necessary for the establishment of the symbiosis with the arbuscular mycorrhiza *Glomus intraradices*. The knockdown of *CgSymRK* was seen to strongly affect penetration of the fungal hyphae into the root cortex, thus revealing the key role of CgSymRK in root endosymbioses in *Casuarina*, and the conservation of SymRK function between legumes and actinorhizal plants (Gherbi et al. 2008a).

5.2 Search for Other Candidates of the Signalling Pathway

Our group has developed the first genomic platform to identify plant genes involved in the symbiotic process between *Frankia* and *C. glauca* (Hocher et al. 2011). A total of 2,028 ESTs was first reported from cDNA libraries corresponding to mRNA extracted from young nodules induced by *Frankia* and from non-infected roots. More recently, a new collection of around 40,000 EST corresponding to 15,000 unigenes was obtained from roots and nodulated roots of *C. glauca* at different stages of the symbiotic process (Hocher et al. 2011). In half of the nodule transcripts, no similarity to previously identified genes was detected, while in the other half, many genes of primary metabolism, protein synthesis, cell division and defence were identified. To explore the early events of *C. glauca-Frankia* symbiosis, a substractive hybridization library (SSH) was also constructed with roots sampled 4 days after infection. A total of 703 SSH sequences were validated and annotated revealing a large proportion of ESTs involved in defence, cell wall structure, and gene expression.

Based on the comparison with legume sequences, ESTs sharing significant homologies with genes from the Nod signalling pathway were identified (Table 2) (Hocher et al. 2011). Functional analyses of the candidate orthologues to the *DMI3* and *NIN* genes are in progress. DMI3 is a calcium and calmodulin-dependent protein kinase that is presumed to decode and transduce Nod-factor-specific calcium spiking

Table 2 Nod-factor signalling genes identified in *Lotus japonicus* (reviewed in Madsen et al. 2010) and homologues EST in *Casuarina glauca*

	L. japonicus	*C. glauca* ESTs
LysM-RLKs	*NFR1*	+
	NFR5	+
LRR-RLK	*SymRK*	*CgSymRK*
Cation channels	*POLLUX*, *CASTOR*	+
Nucleoporins	*NUP85*	–
	NUP133	+
CCaMK	*CCaMK*	+
GRAS-type TFs	*NSP1*	+
	NSP2	+
TF	*NIN*	+

The functional characterization of *CgSymRK* has shown that this actinorhizal gene is functionally equivalent to *SymRK* from legumes (Gherbi et al. 2008). +: EST from *C. glauca* exhibiting significant homologies with genes from the Nod signalling pathway in legumes. *RLK* receptor-like kinase, *TF* transcription factor

response in legumes (Levy et al. 2004; Mitra et al. 2004). NIN (for nodule inception) is a transcriptional regulator that is required for the formation of infection threads and the initiation of nodule primordia. In contrast to DMI3, which is necessary for both nodulation and endomycorrhization, NIN specifically regulates the development of root nodules (Schauser et al. 1999; Marsh et al. 2007). From these data, we consequently expect to determine if the symbiotic signalling pathway is conserved in actinorhizal and rhizobial nitrogen-fixing symbioses beyond the common SYM genes.

6 Gene Expression During Infection

6.1 Actinorhizal Gene Expression

As expected, the transcriptomic approach has shown that many genes are specifically expressed or enhanced in actinorhizal nodules compared with expression in roots (Hocher et al. 2006, 2011). Several authors have already reviewed the plant genes that have been characterized in actinorhizal nodules (Vessey et al. 2005; Laplaze et al. 2008). Here, we focus on recent data concerning plant genes expressed in the root hairs and in the infection zone (zone 2) of the nodule.

During the intracellular infection process, the root hair is the primary site of recognition and infection by the microorganism *Frankia* (Bhuvaneswari and Solheim 2000). Since at present no root-hair-enriched cDNA library is available for any actinorhizal plant, knowledge comes from the characterization of candidate symbiotic genes using promoter-reporter gene approaches. Plant genes whose expression is correlated with root hair invasion by the endosymbiont include two genes from the common Sym pathway, *CgSYMRK* and *CgCCaMK* (our group, unpublished data), and *Cg12*, a gene from *C. glauca* encoding a subtilisin-like serine protease (Laplaze et al. 2000b; Svistoonoff et al. 2003). Subtilases are a superfamily of proteases that are thought to play a role in different aspects of plant development including response to pathogens and lateral root development. P*Cg12* was found to drive expression in infected root hairs and nodule cortical cells containing growing infection threads. This pattern suggests that the subtilase CG12 may play a role in the maturation of a polypetide in the infection threads or in cell wall remodelling resulting from infection by *Frankia*. Interestingly, the study of transgenic *M. truncatula* plants containing the construct P*Cg12-GUS* construct revealed the same pattern of *GUS* expression during the infection process of *M. truncatula* by *Sinorhizobium meliloti* (Svistoonoff et al. 2004). It should also be noted that in absence of a GFP-expressing *Frankia* strain, transgenic P*Cg12-GUS* plants are valuable tools to visualize and count the infection sites during the early stages of the symbiotic process. The use of this approach revealed that in P*Cg12-GUS* plants retransformed with an *RNAi-CgSymRK* construct, the number of infection sites was considerably reduced, thereby establishing the involvement of CgSYMRK in root hair infection.

6.2 *Expression of Heterologous Promoters from Legumes*

To investigate the similarities between nitrogen-fixing symbioses in Rhizobium–legumes and *Frankia*-actinorhizal plants, heterologous promoters from early symbiotic genes of legumes were introduced into actinorhizal plants. Studies concerning the expression pattern conferred by the P*GmEnod40-2-GUS* construct showed that the promoter region from the early symbiotic *Enod40-2* gene from soybean was inactive during actinorhizal nodule development (Santi et al. 2003). In legumes, *Enod40* genes are first expressed in pericycle cells, and later in nodule primordium. They play role in nodule initiation and bacteroid development (Wan et al. 2007).

The heterologous promoter *Enod11* from *M. truncatula* was recently genetically transformed into *C. glauca* (Svistoonoff et al. 2010b). In *M. truncatula*, the *MtEnod11* gene is one of the earliest genes expressed in the root epidermis following contact with *S. meliloti* (Journet et al. 2001; Charron et al. 2004). It encodes a putative cell wall repetitive hydroxy-proline-rich protein and it has proven to be a valuable marker for early infection-related symbiotic events. In legumes reporter gene activity driven by the P*MtEnod11* promoter was observed in the epidermis within the first hours of infection by *S. meliloti* and then in infected root hairs and the infection zone of young nodules. When introduced into *C. glauca*, there was no response in the root epidermis after *Frankia* inoculation during the root perception stage of the unknown actinobacterial factors. P*MtEnod11-GUS* was seen to be expressed as soon as the actinomycete entered a curled root hair, and subsequently in prenodules and the nodule cortical cells being infected by *Frankia*. The expression pattern in nodules was similar to that conferred by the promoter of P*PsENOD12b* (Sy et al. 2006), another early nodulin from *Pisum sativum* also encoding a repetitive proline-rich protein (Vijn et al. 1995). From these data, it can be concluded that there is some conservation of gene regulatory pathways between legumes and actinorhizal plants in cells involved in bacterial infection and accommodation. Conversely, since there is no heterologous expression prior to the penetration of *Frankia* in root hairs, the perception stages appear to involve transcriptional regulation processes that differ in actinorhizal plants (Svistoonoff et al. 2010b).

7 Hormones and Actinorhizal Nodulation

7.1 *Hormones Produced by Frankia*

Bacteria living in association with plants have been shown to produce different phytohormones that could play a role in the symbiotic interaction. Natural auxins such as indole-3-acetic acid (IAA) or phenylacetic acid (PAA) were found in *Frankia* cultures at relatively high concentrations (10^{-5} to 10^{-6} M) (Wheeler and Henson 1979; Wheeler et al. 1984; Hammad et al. 2003). The cytokinin isopentenyl

adenosine (iPA) was also detected at a concentration of 10^{-6} M (Gordons et al. 1988; Stevens and Berry 1988). However, it remains to be established whether or not *Frankia* produces auxin and/or cytokinin *in planta* during the infection process. Transcriptome analyses of the symbiotic form of the actinobacterial genes will likely provide some evidence to elucidate the contribution of the symbiont in the hormonal content of the nodule.

7.2 A Role for Auxin

Auxins are a class of plant growth hormones with many roles in cell division and enlargement, differentiation, lateral root formation, and vascular bundle formation (Casimiro et al. 2003). Since these biological processes occur during actinorhizal nodule formation and because actinorhizal nodules anatomically and ontogenetically resemble lateral roots, the role of auxin in the actinorhizal symbiosis was addressed. Elevated levels of auxins were measured in the actinorhizae (Wheeler and Henson 1979) and application of auxin efflux transport inhibitors such as NPA or TIBA on *C. glauca* roots led to the formation of nodule-like structures called "pseudonodules" on the roots of *Casuarinaceae* plantlets (Duhoux et al. 1996). Attempts to use auxin-sensitive promoters such as the soybean *GH3* promoter (Reddy et al. 2006) or the synthetic DR5 promoter (Ulmasov et al. 1995) to monitor changes either in auxin concentration or in cellular sensitivity during the infection process were unsuccessful.

However, interesting results resulted from the functional analysis of the *CgAUX1*, which encodes a high-affinity auxin influx transporter in *C. glauca*. Using the *GUS* gene driven by the *CgAUX1* promoter, it was clearly shown that auxin plays an important role during plant cell infection in actinorhizal symbiosis (Péret et al. 2007). *CgAUX1-GUS* was found to be strongly expressed both during root hair infection and cortical cell invasion by *Frankia*. It was also reported that *CgAUX1-GUS* was highly expressed in the root primordium, whereas no reporter gene activity was observed in the nodule primordium. This result further indicates that, although the actinorhizal nodule is comparable with a symbiotic lateral root, the molecular mechanisms involved in primordia initiation in lateral roots may differ from those in actinorhizal nodules.

7.3 Cytokinin and Actinorhizal Nodulation

Besides auxin, elevated levels of cytokinins have been found in actinorhizal nodules (Wheeler and Henson 1979) and the induction of bacteria-free nodules, so-called pseudo-actinorhiza, by cytokinin has been reported (Rodriguez-Barrueco and de Castro 1973). To monitor changes in cytokinin levels during the actinorhizal nodulation process, we introduced into Casuarina a transcriptional fusion between

the reporter gene *GUS* and the *Arabidopsis* cytokinin-responsive gene ARR5 (D'Agostino et al. 2000). β-Glucuronidase activity was found to be restricted to the root cap and the root meristems, and no change in expression resulted from infection by *Frankia*, thus implying that this heterologous promoter was not appropriate to study changes in cytokinin levels in Casuarina. More valuable data will probably come from the isolation of cytokinin-responsive genes from actinorhizal plants.

8 Conclusions

Although several actinorhizal genera contain species that are economically important in forestry and land regeneration (Dawson 1986), progress has been slower in our knowledge of the actinorhizal symbiosis than that of the legume–Rhizobium interaction. Major advances have nevertheless been made in recent years thanks to the development of genomics and the transgenic tools. Progress includes the sequencing and comparative analysis of an increasing number of *Frankia* genomes, even though the most decisive progress will come from the development of a genetic approach in *Frankia*. The availability of a method for the genetic transformation of Casuarinas has resulted in a major breakthrough since it opened the way to functional gene analysis in the actinorhizal host plant. It recently led to a major result establishing that the unknown *Frankia* factors, Rhizobial Nod factors and Myc factors use a common pathway (Gherbi et al. 2008a). Deep sequencing analyses are now in progress in actinorhizal plants and mining these data will undoubtly contribute to dissecting the molecular dialogue between *Frankia* and the host plant in the different stages of development of the actinorhizal nodule.

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Signalling in Cyanobacteria–Plant Symbioses

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Abstract Cyanobacteria are a morphologically diverse and widespread group of phototrophic bacteria, many of which are capable of nitrogen fixation. They form symbioses with a wide range of eukaryotic hosts including fungi (lichens and *Geosiphon pyriformis*), diatoms, dinoflagellates, sponges, ascidians (sea squirts), corals and plants. The best understood are the plant symbioses, which are the subject of this chapter. In the cyanobacteria–plant associations, the cyanobacteria provide the host with fixed nitrogen and usually adopt a heterotrophic form of nutrition, using fixed carbon supplied by the plant, enabling them to occupy regions of the host, such as the roots, that receive little or no light. Most cyanobacterial symbionts of plants belong to the genus *Nostoc*, members of which fix nitrogen in specialised cells known as heterocysts, which provide the necessary microoxic environment for the functioning of the oxygen-sensitive enzyme nitrogenase. These cyanobacteria, which are immotile for most of their life cycles, produce specialised motile filaments known as hormogonia, as a means of dispersal and as the infective agents in plant symbioses. Host plants improve their chances of infection by releasing external chemical signals that both stimulate hormogonia formation and serve as chemoattractants. However, within the symbiotic tissue the plant releases hormogonia-repressing factors to ensure the conversion of hormogonia into heterocyst-containing, nitrogen-fixing filaments.

1 Introduction

Cyanobacteria are a large group of morphologically diverse phototrophic bacteria found in almost every environment on Earth. Because of their abundance, especially in the oceans, they make enormous contributions to global carbon and

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nitrogen fixation. Cyanobacteria form symbiotic associations with a wide variety of eukaryotic hosts including plants, fungi, sponges and protists. The cyanobacterial symbionts (cyanobionts) can provide fixed carbon for non-photosynthetic hosts such as the fungi of both lichens (Rikkinen 2002) and *Geosiphon pyriformis* (Kluge 2002; Kluge et al. 2002; Adams et al. 2006), but their primary role is to provide fixed nitrogen, enabling the host to occupy nitrogen-poor environments. They may also protect some hosts, such as ascidians and isopod crustaceans, from grazing by predators, and others, such as sponges, from excessive sunlight (Usher 2008). The benefits to the cyanobionts are less clear but are likely to include protection from competition, predation and environmental extremes. In the case of plants, the cyanobionts can also adapt to a heterotrophic life style using carbon from the plant host, enabling them to devote more resources to the fixation of nitrogen for both partners, and to grow in plant structures, such as the roots of cycads and the stem glands of *Gunnera* that receive little or no light.

Cyanobacteria vary in morphology from simple unicells to complex filamentous forms capable of differentiating several specialised cell types including the nitrogen-fixing heterocyst (Fig. 7c, d) which provides suitable microoxic conditions for the fixation of nitrogen by the oxygen-sensitive enzyme nitrogenase (Meeks et al. 2002; Meeks and Elhai 2002; Golden and Yoon 2003; Zhang et al. 2006; Flores and Herrero 2010). The cyanobionts of plants are filamentous heterocyst-forming cyanobacteria primarily from the genus *Nostoc*. Another important characteristic of these cyanobacteria is the production of specialised, motile filaments known as hormogonia. These provide *Nostoc* spp. (and cyanobacteria of other heterocystous genera such as *Calothrix* and *Fischerella*) with a motile phase and hence a means of dispersal in an otherwise sessile life cycle. Hormogonia are also the infective agent in the plant symbioses (Meeks et al. 2002; Meeks and Elhai 2002; Meeks 2009; Flores and Herrero 2010). Indeed, many plants release unidentified chemicals that stimulate the formation of hormogonia and attract them to the symbiotic tissues (Sect. 3).

2 The Cyanobacteria–Plant Symbioses

This chapter deals with the endophytic cyanobacteria–plant symbioses in which the cyanobiont is located within the host tissue or, in the case of the angiosperm *Gunnera*, within the host cells. However, many cyanobacteria, including nitrogen-fixing strains, grow as epiphytes on a wide range of plants, particularly in aquatic environments (Adams 2011). These "loose" associations won't be discussed here, as they are poorly studied and little is known about the extent to which the plant benefits, although the nitrogen-fixing cyanobacteria certainly contribute to the local nitrogen economy. By contrast, in the endophytic associations the plant host clearly benefits from the provision of combined nitrogen by the cyanobionts and there are clear, although not well understood, interactions between the cyanobiont and its host.

2.1 Bryophytes (Mosses, Hornworts and Liverworts)

The mosses, hornworts and liverworts are small, non-vascular land plants (Figs. 1 and 3a), some of which can become infected with primarily heterocystous cyanobacteria (Adams 2002a, b; Meeks 2003; Solheim et al. 2004; Adams et al. 2006; Adams and Duggan 2008; Bergman et al. 2007a, 2008). The cyanobacteria in moss associations are mostly epiphytic (Solheim and Zielke 2002; Solheim et al. 2004; Gentili et al. 2005), except for two *Sphagnum* species in which they occupy water-filled, dead (hyaline) cells, where they may gain protection from the low pH of the bog environment (Solheim and Zielke 2002). The most common cyanobacterial epiphytes on mosses are members of the filamentous, heterocystous genera *Nostoc*, *Stigonema* and *Calothrix* (DeLuca et al. 2002, 2007; Gentili et al. 2005; Houle et al. 2006), although the non-heterocystous, filamentous genera *Phormidium* and *Oscillatoria*, and even the unicellular *Microcystis*, have also been reported (Solheim et al. 2004). These moss associations will not be discussed further here as they are almost exclusively epiphytic, and even in the seemingly endophytic *Sphagnum* symbioses the cyanobacteria occupy dead cells which are connected to the outside environment. Nevertheless, these nitrogen-fixing cyanobacteria–moss associations are of environmental significance as they are often the major source of combined nitrogen in local ecosystems in the Arctic, the Antarctic and forests of the northern hemisphere where mosses are abundant (Zielke et al. 2002, 2005; Solheim and Zielke 2002; Nilsson and Wardle 2005; DeLuca et al. 2008).

Fig. 1 The liverwort *Blasia pusilla* collected from the wild, showing the dark *Nostoc* colonies (~0.5–1.0 mm in diameter) bordering the thallus midrib. Reproduced with permission from Adams (2000)

Endophytic cyanobacterial symbioses are found in all 13 hornwort genera (Renzaglia et al. 2007), but in only two (*Blasia* and *Cavicularia*) of the greater than 340 liverwort genera, although a further two (*Marchantia* and *Porella*) form epiphytic associations (Rai et al. 2000; Adams 2000, 2002a, b; Adams et al. 2006; Adams and Duggan 2008). In the endophytic symbioses, cyanobacterial colonies can be seen as dark spots up to 0.5 mm in diameter within the flattened thallus of the plant (Fig. 1). The endophytes of liverworts and hornworts are almost exclusively members of the genus *Nostoc* (Costa et al. 2001; Rasmussen and Nilsson 2002; Adams 2002a, b; Adams et al. 2006; Bergman et al. 2007a; Adams and Duggan 2008), and a wide variety of strains can infect a single thallus in the field (West and Adams 1997; Costa et al. 2001; Adams 2002a, b; Adams and Duggan 2008), although some can be dominant and widespread (Rikkinen and Virtanen 2008). The ease with which liverworts and hornworts can be grown in the laboratory in shaken liquid culture (Fig. 2b), with or without symbiotic cyanobacteria, makes them an excellent model for studying the infection process (Adams 2002a, b; Meeks 2003; Duckett et al. 2004; Adams and Duggan 2008).

The structures housing the symbiotic cyanobacteria in hornworts (slime or mucilage cavities; Fig. 2a) and liverworts (auricles; Fig. 2c, d) are produced constitutively and not as a response to the presence of potential symbionts (Renzaglia et al. 2000; Adams 2002a, b; Meeks 2003; Adams et al. 2006; Villarreal and Renzaglia 2006; Bergman et al. 2008). The slime cavities of hornworts such as *Anthoceros* and *Phaeoceros* are formed within the thallus and are connected to the ventral surface by mucilage clefts (Fig. 2a) which superficially resemble stomata, but are not thought to be related (Villarreal and Renzaglia 2006). The cleft results from the separation of adjacent epidermal cells, after which the slime cavity develops beneath the cleft (Renzaglia et al. 2000). The slime cavities in the hornwort *Leiosporoceros dussii* also develop beneath mucilage clefts (Fig. 3c, d) but are elongated mucilage-filled "canals" (Fig. 3b) resulting from the separation of plant cell walls along their middle lamellae. These canals branch to form an integrated network enabling the *Nostoc* symbiont to spread throughout the thallus (Villarreal and Renzaglia 2006). By contrast, in the liverwort *Blasia* the cyanobacteria occupy dome-shaped auricles (Fig. 2c, d), which develop on the ventral surface of the thallus from a three-celled mucilage hair that undergoes extensive elaboration (Renzaglia et al. 2000).

2.2 Gymnosperms (Cycads)

Cycads are evergreen palm-like plants, from 10 cm to 20 m in height, which once dominated the Earth's forests but are now restricted to subtropical and tropical regions of mostly the southern hemisphere (Brenner et al. 2003; Vessey et al. 2005). Cycads produce coralloid (coral-like) roots (Fig. 4a) that become infected with heterocystous cyanobacteria (Costa and Lindblad 2002; Lindblad and Costa 2002; Vessey et al. 2005; Lindblad 2009) usually from the genus *Nostoc*, although

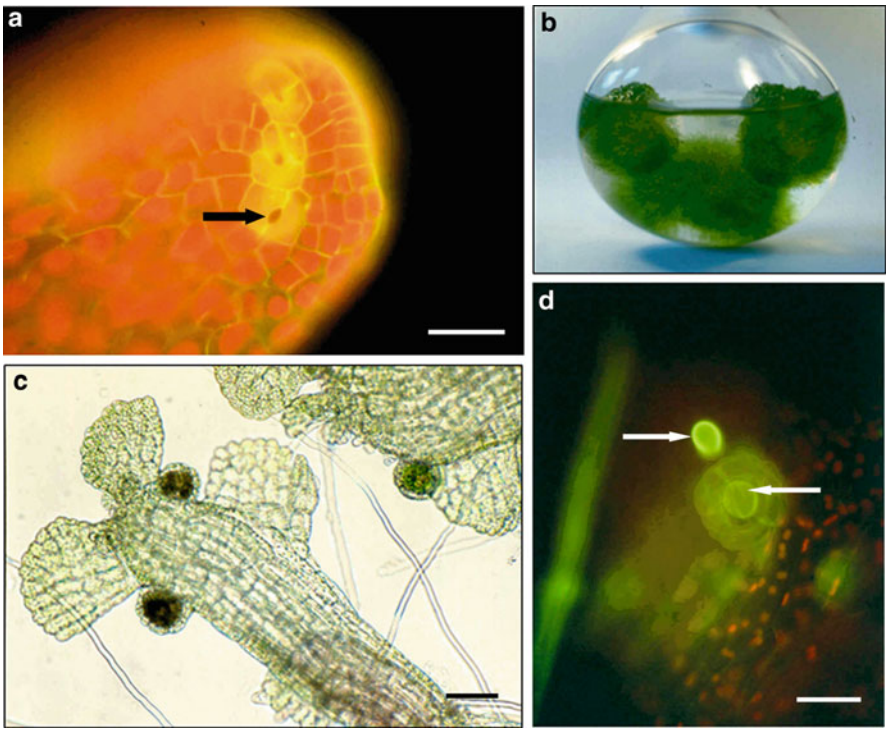

Fig. 2 The liverwort and hornwort symbioses. (**a**) Fluorescence micrograph of the hornwort *Phaeoceros* sp. stained with calcofluor, showing the slit-like entrances (one of which is arrowed) through which hormogonia gain entry to the slime cavities beneath. (**b**) View of the underside of an Erlenmeyer flask containing the liverwort *Blasia pusilla* grown free of cyanobacteria in shaken liquid medium. (**c**) *Blasia pusilla* growing in liquid culture showing three auricles infected in the laboratory with two different *Nostoc* strains, one brown pigmented (the two auricles to the left) and the other blue-green. (**d**) Fluorescence micrograph of uninfected *Blasia* stained with calcofluor. A single auricle can be seen with one inner (*lower arrow*) and one outer (*upper arrow*) slime papilla. Bars 50 μm. Photographs (**a**) and (**d**) courtesy of S. Babic. (**a**) and (**d**) reproduced with permission from Adams (2000) (**b**) reproduced with permission from Adams (2002a) (**c**) reproduced with permission from Adams and Duggan (1999)

Calothrix spp. have been reported on a number of occasions (Costa and Lindblad 2002; Rasmussen and Nilsson 2002; Bergman et al. 2007a, 2008; Gehringer et al. 2010; Thajuddin et al. 2010). Single or multiple strains can infect a coralloid root (Zheng et al. 2002; Costa et al. 2004; Thajuddin et al. 2010) although some *Nostoc* spp. can be dominant and, at least in the cycad genus *Macrozamia*, there seems to be little host specialisation by cyanobionts in the field (Gehringer et al. 2010).

Coralloid roots grow out from the tap root, and then upwards, sometimes breaking the soil surface. The mechanism by which the cyanobacteria enter the roots is unknown, although suggestions include lenticels and breaks in the dermal layer (Costa and Lindblad 2002; Vessey et al. 2005; Bergman et al. 2007a) possibly resulting from degradation of the cell wall caused by bacteria and fungi in the cycad

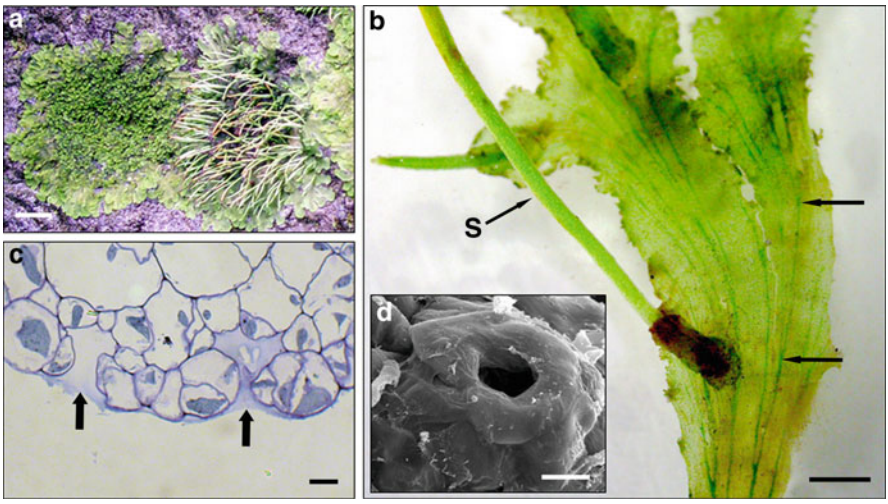

Fig. 3 The hornwort *Leiosporoceros dussii* with symbiotic *Nostoc*. (**a**) To the left is a young rosette and to the right an older thallus with numerous upright sporophytes. (**b**) Dark green *Nostoc* "strands" (*arrows*) can be seen within the thallus, parallel to the main axis. *S* sporophyte. (**c**) Light micrograph of a nearly transverse section of two mucilage clefts (*arrows*), which provide the entry point for cyanobacterial infection; the *Nostoc* filaments subsequently spread through channels created by the separation of cells along their middle lamellae. (**d**) Scanning electron micrograph of a mucilage cleft. Bars 10 mm in (**a**), 2 mm in (**b**), 15 μm in (**c**) and 20 μm in (**d**). Reproduced with permission from Villarreal and Renzaglia (2006)

rhizosphere (Lobakova et al. 2003). The cyanobionts occupy a mucilage-filled zone between the inner and outer cortical layers (Fig. 4b). Although coralloid roots develop in the absence of cyanobacteria, infection induces morphological alterations (Sect. 5.4) which increase the area of contact between plant cells and the cyanobiont, to enhance nutrient exchange (Adams 2000; Rai et al. 2000; Costa and Lindblad 2002; Lindblad 2009).

2.3 Angiosperms (Gunnera)

This is the only intracellular cyanobacterial-plant symbiosis (the *Nostoc* cells are found between the host cell wall and the plasmalemma) and the only symbiosis with a flowering plant (Bergman 2002; Bergman and Osborne 2002; Bergman et al. 2007a). The genus *Gunnera* consists of plants with leaves varying from 1 cm to several metres across (Fig. 5a), once found only in warm, wet equatorial regions, but which now commonly occur in suitably wet temperate regions (Osborne and Sprent 2002). The cyanobiont is found in mucus-secreting stem glands (Figs. 5b and 6a, b) and supplies the entire nitrogen requirements of even the largest plants (Bergman 2002; Bergman et al. 2007a). In the field, *Gunnera* is infected by a wide range of *Nostoc* spp. (Nilsson et al. 2000; Rasmussen and Svenning 2001; Guevara et al. 2002; Svenning et al. 2005) all of which are capable of high levels of

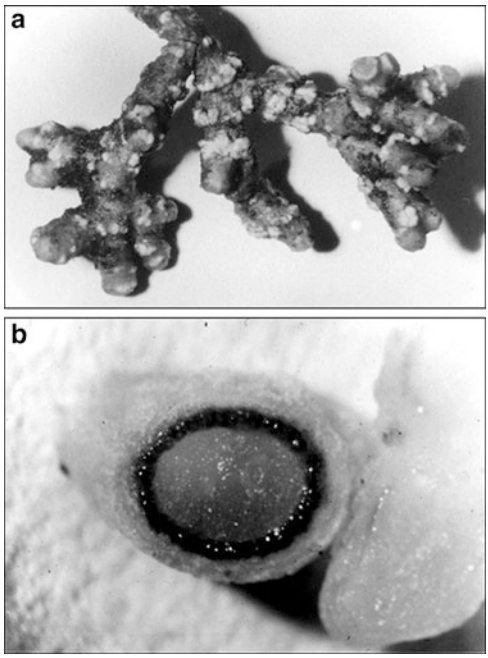

Fig. 4 The cycad–*Nostoc* symbiosis. (**a**) Cycad coralloid root, which is the site of cyanobacterial infection. (**b**) Transverse section of the root showing the dark cyanobacterial band between the inner and outer cortical layers of the root. (**a**) Reproduced with permission from Lindlbad et al. (1985) (**b**) reproduced with permission from Rai et al. (2000)

differentiation into hormogonia (Bergman et al. 2007a, 2008). A single stem gland may occasionally contain several different (but closely related) strains of *Nostoc* (Nilsson et al. 2000). However, even closely related *Nostoc* strains can differ in their ability to infect *Gunnera* (Papaefthimiou et al. 2008a) and some strains that form large numbers of motile hormogonia are incapable of infecting (Nilsson et al. 2006), implying that the successful establishment of the symbiosis involves additional factors, perhaps including chemical signalling between the host and potential cyanobionts (Sect. 4).

Cyanobacteria gain entry to *Gunnera* through the specialised stem glands found at the base of each leaf stem (petiole; Fig. 6a, b). Mucilage secreted by these glands contains unidentified chemical signals that both induce the formation of hormogonia and attract them by chemotaxis (Bergman 2002; Bergman et al. 2007a). The formation of glands is not reliant on the presence of cyanobacteria, although it is stimulated by nitrogen starvation (Chiu et al. 2005). The gland consists of a central papilla surrounded by a further 5–8 papillae, creating narrow channels that lead downwards into the stem tissues (Fig. 6b). Destruction of these channels by removal of all of the outer papillae prevents cyanobacterial infection, but this is restored by leaving just a single outer papilla (Uheda and Silvester 2001). The channels therefore provide the route by which *Nostoc* hormogonia migrate towards the plant tissue at the base of the gland, and once there, they enter the *Gunnera* cells by an unknown mechanism; following cell invasion the host cell wall appears normal (Bergman 2002; Bergman et al. 2007a). Symbiotically competent

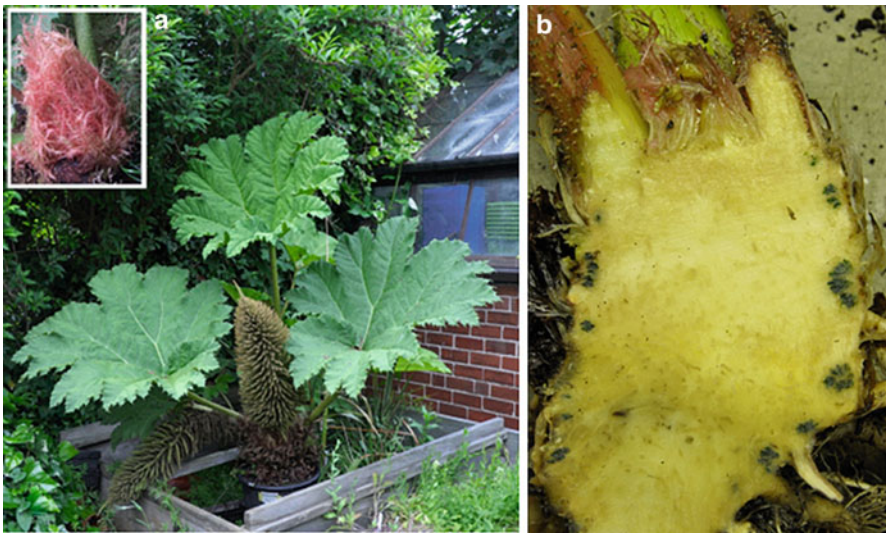

Fig. 5 *Gunnera manicata*. (**a**) A young plant with two large flower spikes. Inset: *Red* pigmented fronds cover the crown of the plant (hidden in the large image), where new leaves and new stem glands develop. (**b**) Vertical cross-section of a rhizome of *Gunnera manicata*. Cyanobacterial colonies (0.5–2 cm in diameter) can be seen as green patches around the periphery of the rhizome. New leaves will develop in the region between the two leaf petioles at the top of the image, which is an area covered by red fronds (see inset in **a**). New stem glands form close to the base of each newly developing leaf petiole and subsequently become infected by *Nostoc*. Photos: Owen Jackson

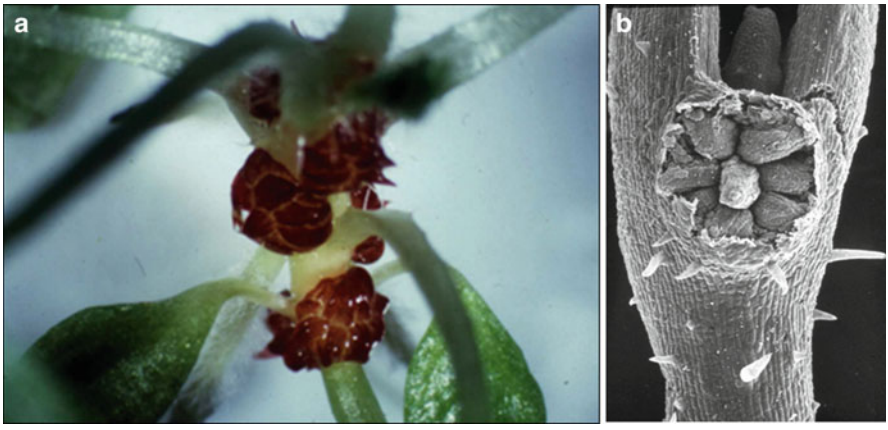

Fig. 6 *Gunnera* stem glands. (**a**) *Gunnera* seedling showing the red stem glands at the base of the leaf petioles. The glands are the entry point for cyanobacteria. (**b**) Scanning electron micrograph of a *Gunnera chilensis* gland showing the arrangement of papillae. Hormogonia gain entry into the internal stem gland tissue by migrating down the channels between the papillae. (**a**) reproduced with permission from Adams et al. (2006) (**b**) reproduced with permission from Bergman et al. (1992)

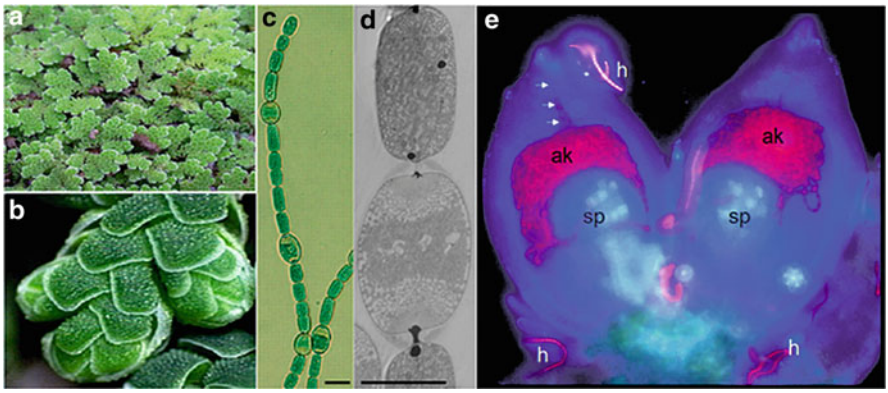

Fig. 7 The water fern *Azolla*. (**a**) View from above, of *Azolla filliculoides* floating on the water surface. (**b**) View of an *Azolla* branch showing the overlapping dorsal lobes of the leaves which contain the cyanobionts. (**c**) Light micrograph of the *Azolla* cyanobiont with the large heterocysts clearly visible. (**d**) Transmission electron micrograph of a thin, longitudinal section of a cyanobiont filament showing a heterocyst (*centre*) with a vegetative cell on either side. (**e**) Fluorescence micrograph of a pair of megasporocarps (*blue*) which become infected with cyanobacteria when the motile hormogonia (h) on the surface, enter via channels (*arrows*). Once inside the megasporocarp the cells of the hormogonia convert into a form of spore called an akinete (ak) which can be seen as the intensely fluorescing area (*red*) above the megaspores (sp). These akinetes provide the inoculum for the next generation of the fern, so maintaining the continuity of the symbiosis. Bars 5 μm (**c**), 5 μm (**d**). Reproduced with permission from Ran et al. (2010)

cyanobacteria are known to produce the phytohormone indole-3-acetic acid (Sergeeva et al. 2002; Liaimer and Bergman 2004) which might be responsible for localised mitotic activity of the host cells which are thought to be the site of infection (Bergman et al. 2007a). The gland channels disappear at this stage, preventing any further infection. The cyanobacteria fill the *Gunnera* cells but never enter the cytoplasm as they are confined between the plasmalemma and the cell wall, the former acting as the symbiotic interface where nutrients are exchanged between the partners (Bergman 2002; Bergman et al. 2007a).

2.4 Pteridophytes (Azolla)

Water ferns of the genus *Azolla* have overlapping leaves consisting of two 1-mm long lobes (Fig. 7a, b), the upper one containing diazotrophic cyanobacteria in an ovoid cavity approximately 0.3 mm in length, and the lower achlorophyllous lobe serving as a float to keep the upper lobe above the water surface (Adams 2000; Lechno-Yossef and Nierzwicki-Bauer 2002; Carrapico 2002; van Hove and Lejeune 2002a, b; Bergman et al. 2007a, b). These ferns are generally no larger than 3–4 cm and are found on still or slow-moving freshwater bodies in temperate

to tropical climates. The association has been used for centuries as a green manure for rice cultivation (Vaishampayan et al. 2001; Bergman et al. 2007a, b) but also has potential applications in mosquito control and as a supplemental animal feed (van Hove and Lejeune 2002a, b; Choudhury and Kennedy 2004).

Azolla is unique among the cyanobacteria–plant symbioses in being the only example in which the cyanobiont is perpetually associated with the host, being transferred from generation to generation. Mature dorsal leaf cavities of *Azolla* contain approximately 2,000–5,000 cyanobiont cells together with a complex community of heterotrophic bacteria, all of which are immobilised within a polysaccharide-rich mucilage around the periphery of the cavity, the central region being gas-filled (van Hove and Lejeune 2002a, b; Lechno-Yossef and Nierzwicki-Bauer 2002). The wide range of bacteria found in the leaf cavities (and other parts of the plant) are mostly unculturable (Bergman et al. 2007a; Zheng et al. 2009a, b). Like the cyanobiont, these bacteria are present throughout the life cycle of the plant and may therefore play an important role in the association, perhaps by contributing to nitrogen fixation and the production of the polysaccharide-rich mucilaginous matrix associated with the leaf cavities (Zheng et al. 2009a, b), or by releasing the plant hormone auxin (Lechno-Yossef and Nierzwicki-Bauer 2002).

The *Azolla* leaf cavity is occupied by an abundant primary cyanobiont (Fig. 7c, d), which appears to be unable to grow outside the symbiosis, and a much less abundant secondary cyanobiont which can be readily cultured (Papaefthimiou et al. 2008a, b; Sood et al. 2008a, b). These major and minor cyanobionts show significant phenotypic differences. In 1873, Strasburger was the first to refer to the major cyanobiont as *Nostoc*, but this was later changed to *Anabaena*, since when there has been continuing controversy about its correct generic assignment. The major cyanobiont is referred to as *Nostoc azollae* 0708 in the recently published draft genome sequence (http://genome.jgi-psf.org/anaaz/anaaz.home.html; Ran et al. 2010), although the closest phylogenetic relatives appear to be *Raphidiopsis brookii* D9 and *Cylindrospermopsis raciborskii* CS-505, which have the smallest sequenced genomes of the filamentous cyanobacteria. However, *Nostoc azollae* 0708 shares the highest number of protein groups exclusively with the symbiotically competent *Nostoc punctiforme* PCC 73102 (Ran et al. 2010). The primary cyanobiont has a small (5.49 Mb) genome, comprising one chromosome and two plasmids, and contains 5,357 coding sequences of which 3,668 have intact open reading frames, while the rest are pseudogenes. High numbers of pseudogenes are a trait associated with endosymbionts in sheltered environments where the likelihood of encountering foreign DNA is low. The large proportion of pseudogenes (31.2%) found within all genomic functions present in the genome of the cyanobiont suggests a high level of gene erosion (Ran et al. 2010).

The permanent association between *Azolla* and its cyanobiont is maintained by the transfer of the cyanobacteria to the progeny of both asexual and sexual reproduction. The former is the main form of reproduction and involves the doubling of plant biomass in approximately 2 days, accompanied by fragmentation of branches from the main stem. Cyanobionts, in the form of hormogonia-like, non-heterocystous filaments, are transferred to the cavities of newly developing leaves at the apical meristem, with the help of bridge-like structures formed by primary branched

hairs (Zheng et al. 2009a, b). Sexual reproduction of the fern seems to be triggered by adverse environmental conditions (reviewed by Lechno-Yossef and Nierzwicki-Bauer 2002; Pabby et al. 2004a) and involves sporophytes that produce male microsporocarps and female megasporocarps, the latter containing a single megasporangium. Within the indusium chamber of each megasporangium is a single megaspore and megaspore apparatus, together with a colony of the cyanobiont which, in the form of hormogonia-like filaments, entered the chamber via a pore at the top (Fig. 7e; Zheng et al. 2009b). The pore subsequently closes and the cells of the filaments undergo synchronous conversion to spore-like resting cells known as akinetes (Zheng et al. 2009b). In this way the mature, dormant megasporocarp contains similarly dormant cyanobiont akinetes. Megasporocarp fertilisation and embryogenesis are followed by akinete germination to form active cyanobiont filaments that provide the inoculum for the embryonic leaf (Zheng et al. 2009a).

3 The Role of Hormogonia in Plant Infection

3.1 Hormogonia and Motility

The primary function of the cyanobiont in plant symbioses is to provide combined nitrogen for the partnership and, accordingly, they are mostly heterocystous cyanobacteria, primarily from the genus *Nostoc*. For cyanobacteria in the soil to find a suitable plant host they must be motile, yet members of the genus *Nostoc* are non-motile for most of their life cycle. However, they can produce short, motile filaments known as hormogonia which serve both as a means of dispersal and as the agent of plant infection (Meeks 2003, 2009; Adams 2000; Gusev et al. 2002; Meeks et al. 2002; Meeks and Elhai 2002; Bergman et al. 2007a). Hormogonia development is triggered by a variety of environmental factors (Meeks et al. 2002; Meeks and Elhai 2002; Meeks 2009) including chemicals released by plants (Sect. 4). The process begins with a round of rapid, synchronous cell divisions in all vegetative cells, followed by filament fragmentation at the junction between each heterocyst and the neighbouring vegetative cell. This releases free heterocysts (which are no longer viable) and short filaments which become the motile hormogonia. After a period of dispersal the hormogonia lose motility and develop heterocysts once more, enabling them to fix nitrogen; this is vital for a stable symbiosis and plants secrete chemicals into the symbiotic tissues to prevent further hormogonia formation.

Even the *Azolla* cyanobiont, which is retained by the fern from one generation to the next, produces hormogonia-like filaments to ensure the transfer of the cyanobiont to the megaspore during sexual reproduction, and to the new leaf cavities that form following asexual reproduction (Sect. 2.4; Zheng et al. 2009a, b). However, members of hormogonia-forming genera other than *Nostoc* are either rarely (e.g. *Stigonema* and *Calothrix*) or never (e.g. *Fischerella*) found as plant symbionts,

implying that additional factors are required for symbiotic competence. One such factor is the ability to sense and react to plant compounds that act as chemoattractants, guiding the hormogonia to the plant structures that will house them. This is likely to be especially important in structures that receive no light, because the natural phototaxis of hormogonia must be overridden to ensure successful infection. A second factor is the ability to adhere to and possibly to specifically recognise the plant surface; for this, external filamentous protein structures known as type IV pili are thought to be involved (see below). Finally, plant cyanobionts need to be facultative heterotrophs to enable them to survive in darkness in cycad roots and *Gunnera* stem glands where they can not photosynthesise, but are provided with fixed carbon by the plant.

3.2 Pili

The surface of *Nostoc* hormogonia is covered by abundant type IV pili (Tfp; Fig. 8). Tfp are found in a wide range of bacteria where they have roles in adhesion, DNA uptake, pathogenesis and motility (Mattick 2002; Nudleman and Kaiser 2004; Burrows 2005). They are also involved in motility in some unicellular cyanobacteria (Bhaya 2004), and their presence on the surface of motile hormogonia, but absence from non-motile vegetative filaments, has led to the suggestion that they are involved in the gliding motility of hormogonia (Duggan et al. 2007). The *N. punctiforme* genome carries homologues of many of the genes known to be involved in pilus biogenesis and function in other bacteria (Meeks et al. 2001) and at least one of these, *pilQ*, is strongly upregulated during hormogonia formation (Klint et al. 2006). In addition, mutation of *pilT* and *pilD*, thought to be involved in Tfp function, greatly decreases the ability of the mutant hormogonia to infect the liverwort *Blasia* (Duggan et al. 2007). However, it is not clear if this is due to loss of motility (and hence, chemotaxis) or some other potential function of the pili, such as adhesion to, or recognition of the plant surface (Duggan et al. 2007).

3.3 Chemotaxis

The importance of signal sensing and chemotaxis in hormogonia is reflected in the dramatic changes in gene expression that accompany hormogonia development induced by either combined nitrogen starvation or hormogonia inducing factor (HIF, Sect. 4; Campbell et al. 2007, 2008). In the first 24 h of *N. punctiforme* hormogonia development, the transcription of 944 genes is upregulated and 856 downregulated (Campbell et al. 2007). A majority of the former encode proteins for signal transduction and transcriptional regulation, with others encoding proteins

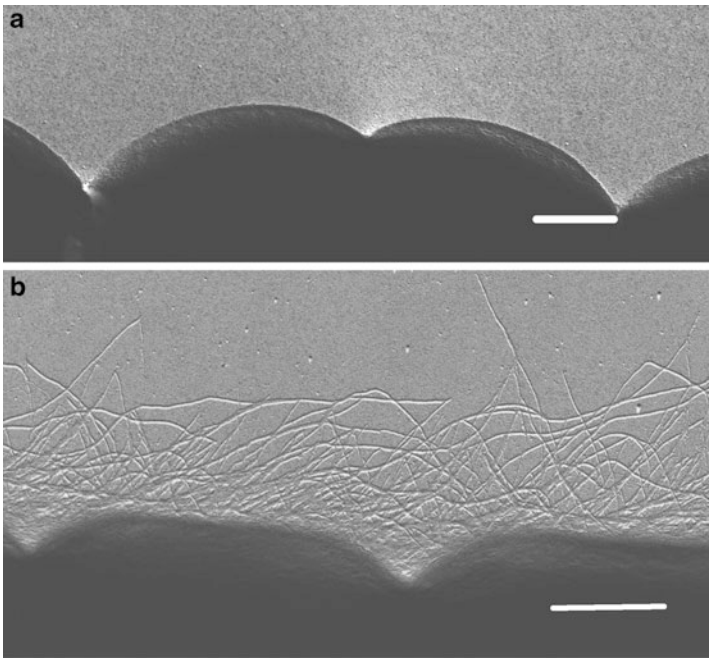

Fig. 8 Comparison of the cell surfaces of wild-type vegetative filaments and hormogonia of the symbiotic cyanobacterium *Nostoc punctiforme*. (**a**) Wild-type vegetative filaments; (**b**) wild-type hormogonia. Scale bars represent 1 μm. For electron microscopy, platinum wire (2 cm by 0.2 mm) was evaporated onto the surface of each sample by using an Edwards 306A high-vacuum coating unit and samples were viewed on a JEOL1200EX transmission electron microscope at 80 kV. Reproduced with permission from Duggan et al. (2007)

with putative roles in chemotaxis and pilus biogenesis (Meeks et al. 2001; Klint et al. 2006; Campbell et al. 2007). Similarly, the genomes of symbiotic cyanobacteria reflect the importance of signal sensing and transduction. For example, the *N. punctiforme* genome has 3–5 copies of genes encoding homologues of chemotaxis-related proteins CheA, CheB, CheW, CheD and CheR (Meeks et al. 2001). Similarly, preliminary annotation of the draft genome sequence of the *Azolla filiculoides* cyanobiont (http://genome.jgi-psf.org/anaaz/anaaz.home.html) has revealed a large number of genes with potential involvement in signal perception and transduction, in addition to genes encoding a possible pilus-related motility apparatus.

3.4 Other Characteristics

In addition to the more obvious hormogonia traits of motility, chemotaxis and plant recognition/adhesion, there is evidence that more subtle aspects of their behaviour

are also crucial for successful plant infection. For example, in *N. punctiforme* inactivation of the gene *sigH*, which encodes an alternative sigma subunit of RNA polymerase, and transcription of which is induced by HIF (Meeks 2003), has no effect on the frequency of HIF-induced hormogonia, yet the hormogonia are fivefold more infective of the hornwort *Anthoceros* than are wild-type hormogonia (Meeks and Elhai 2002; Meeks 2003); the reason for this is unknown.

The infection frequency of *N. punctiforme* hormogonia in the liverwort *Blasia* is also influenced by mutations in *cyaC*, which encodes adenylate cyclase, the enzyme responsible for the biosynthesis of adenosine 3′, 5′-cyclic monophosphate (cAMP), implying that this intracellular messenger may play a role in infection (Adams and Duggan 2008; Chapman et al. 2008). However, the CyaC adenylate cyclase is a multidomain enzyme and mutations in different domains result in hormogonia with wildly different infection frequencies in *Blasia*, with one showing a 300–400% greater infection frequency than the wild type, and another showing only 25% of the frequency of the wild type (Chapman et al. 2008). These different infection phenotypes seem not to be the result of differences in cellular cAMP levels, as these are 25% of wild type in both mutants. Nor do the mutants show any differences in the frequency, motility or piliation of hormogonia induced in the presence of *Blasia*. It seems, therefore, that differences in symbiotic competency result from subtle, unknown shifts in the behavioural characteristics of the mutant hormogonia in response to plant signals.

4 Signalling Between Potential Partners

Host plants increase their chances of infection by releasing compounds that trigger hormogonia formation (hormogonia inducing factor, HIF) and others that act as chemoattractants. Since hormogonia only remain infective while motile, plants can further improve their chances of infection by prolonging the hormogonial (motile) phase. For example, *Gunnera* stem gland mucilage contains a factor(s) that lengthen this motile stage from 20–40 h to several weeks (see Bergman et al. 2007a). HIFs are produced by the hornwort *Anthoceros punctatus* (Meeks et al. 2002; Meeks and Elhai 2002; Meeks 2003, 2009), cycads and *Gunnera* (Rai et al. 2000; Bergman et al. 2008) and the liverwort *Blasia* (Watts 2000; Adams 2002a, b; Adams and Duggan 2008), although none has been chemically identified and they may differ in different plants. The production or release of HIF seems to be stimulated by nitrogen starvation (Meeks and Elhai 2002; Meeks 2003, 2009). The cyanobiont's ability to respond to plant HIF influences the subsequent infection frequency. For example, *N. punctiforme* mutants that show a greater hormogonia induction in response to *Anthoceros* HIF also infect the plant at a greater initial frequency than the wild type. Conversely, mutation of the *N. punctiforme* gene *ntcA*, which encodes the global transcriptional regulator NtcA (Herrero et al. 2004), reduces the frequency of HIF-induced hormogonia, and those that do form fail to infect *Anthoceros* (Wong and Meeks 2002).

Having stimulated hormogonia formation by the release of HIF a plant host must then attract the hormogonia by the production of chemoattractants. A good example is the liverwort *Blasia*, which releases a chemoattractant when nitrogen starved (Knight and Adams 1996; Watts 2000; Adams and Duggan 2008). However, such chemoattractants are not restricted to host plants as they are also produced by non-host plants such as *Trifolium repens* (Nilsson et al. 2006) and germinating wheat seeds (Knight and Adams 1996; Watts 2000; Adams and Duggan 2008). None of these chemoattractants have been chemically identified although they are thought to be sugar-based molecules (Watts 2000); indeed, simple sugars such as arabinose, glucose and galactose have been shown to attract hormogonia, with arabinose being the most effective (Nilsson et al. 2006).

The polysaccharide-rich mucilage secreted by mature stem glands of *Gunnera chilensis* contains polymers of arabinose, galactose and glucuronic acid, with the major form being arabinogalactans associated with arabinogalactan proteins (AGPs; Rasmussen et al. 1996). AGPs are a diverse group of proteoglycans generally found in the extracellular matrix of plant and some algal cells (Gaspar et al. 2001; Seifert and Roberts 2007; Showalter 2001). Although AGPs are found in *Gunnera* stem gland mucilage, their significance for the symbiosis is not known (see: Bergman 2002; Bergman et al. 2007a). However, they have been implicated in the *Alnus–Frankia* symbiosis in which they are found at the symbiotic interface in the root nodules (Berry et al. 2002). *Gunnera* mucilage contains high levels of arabinose, possibly released from AGPs or arabinan-containing pectins by the extracellular enzyme ARAf, expression levels of which are slightly higher in stem tissue containing glands compared with that lacking glands (Khamar et al. 2010).

The high levels of sugars such as arabinose in *Gunnera* mucilage ensure effective chemoattraction of hormogonia into the gland channels, and from there into the deeper tissues. This is aided by the very low levels of the reducing sugars glucose, fructose and sucrose, which are known to suppress hormogonia formation (Khamar et al. 2010). However, these sugars are found at 100-fold higher concentrations in the mature gland tissue from which the mucilage originates, ensuring that the development of hormogonia that were attracted by arabinose in the mucilage and gland channels, is suppressed in mature gland tissue, facilitating heterocyst development and nitrogen fixation. The higher levels of glucose and fructose compared with sucrose in mature gland tissue may result from the high expression levels of genes encoding sucrose-hydrolyzing enzymes, such as cell wall invertase, in stem tissue containing glands compared with tissue lacking glands (Khamar et al. 2010). Although starch is abundant in the cortical cells of stems of nitrogen-starved *Gunnera* seedlings, mature gland tissue is devoid of starch, perhaps as a result of the increased expression of genes encoding enzymes for starch degradation, including starch phosphorylase and α-amylase (Khamar et al. 2010).

In the *Gunnera* symbiosis, stem gland mucilage contains a compound(s) that stimulates expression of *Nostoc* genes *hiaA*, *hiaB* and *hiaC* which encode, respectively, an outer membrane glycoprotein, a putative signalling molecule and a protein that may be involved in the adaptation of *Nostoc* to an acidic environment such as that in stem gland mucilage (Liaimer et al. 2001; Bergman et al. 2007a).

However, as might be expected, the expression of many more *Nostoc* genes is altered in planta. For example, proteomic analysis has identified 38 *Nostoc* proteins differentially expressed in symbiosis, including four upregulated cell surface-associated proteins one of which contains fasciclin-like repeats (Ekman et al. 2006). Such fasciclin domains are typical of surface-associated proteins involved in cell adhesion, which are thought to be symbiotically relevant in several unrelated symbioses including *Nostoc*-containing lichens (Paulsrud and Lindblad 2002).

Another group of signalling compounds with potential involvement in the cyanobacterial symbioses is the lectins. These are produced by the plant host in bryophyte and *Azolla* symbioses, and can bind to sugars on the surface of symbiotic *Nostoc* strains (Lehr et al. 2000; see also: Rai et al. 2000; Adams 2000; Rikkinen 2002; Adams et al. 2006). Little is known about the importance of lectins in cyanobacteria–plant symbioses, although they have been suggested to be involved in fungus-partner recognition in lichens (Lehr et al. 2000; Elifio et al. 2000; Rikkinen 2002; Legaz et al. 2004; Sacristan et al. 2006). The majority of lichens consist of a fungus and a green alga, but around 10% contain a cyanobacterial partner either as sole photobiont, or in combination with a green alga (Rikkinen 2002; Adams 2011). In the cyanolichens *Peltigera canina* and *Leptogium corniculatum,* a fungal arginase acts as a lectin by binding to a polygalactosylated urease in the cell wall of the *Nostoc* cyanobiont (Diaz et al. 2009; Vivas et al. 2010). Lectin binding may also be important in the unique cyanobacteria–fungus association *G. pyriformis* (Adams et al. 2006), as mannose-specific lectin ConA shows changes in its binding during the *Nostoc* life cycle, with strong binding only to so-called primordia, which are hormogonia that have lost motility and are beginning to develop heterocysts (Kluge et al. 2002; Adams et al. 2006). Primordia are also the only stage which can be engulfed by the fungus to establish the symbiosis, implying that *Nostoc* extracellular glycoconjugates could be important in recognition.

5 Host–Cyanobiont Interactions Post-infection

Cyanobacteria in symbiosis with plants generally show morphological modifications such as the cell enlargement and shape irregularity seen in hornwort-associated *Nostoc* (Meeks and Elhai 2002) and in the cyanobiont of *Azolla* (Pabby et al. 2003, 2004b; Papaefthimiou et al. 2008a; Sood et al. 2008a, b; Zheng et al. 2009a). Further changes include repression of hormogonia development, reduction of cell division, increases in heterocyst frequency and nitrogen fixation, and repression of nitrogen assimilation and photosynthetic CO_2 fixation. Such changes are often least pronounced in the youngest tissue (the most recently infected) and greatest in the oldest symbiotic tissues.

5.1 Repression of Hormogonia Development

Although hormogonia are essential for a cyanobiont to locate and invade a plant host, they lack heterocysts and are therefore incapable of nitrogen fixation, so their formation must be repressed once the cyanobiont is inside the host. In the hornwort *A. punctatus,* the plant achieves this repression by releasing into the symbiotic cavity an unidentified water-soluble hormogonia-repressing factor (HRF) which is dominant over HIF (Cohen and Yamasaki 2000; Meeks and Elhai 2002; Meeks et al. 2002; Campbell et al. 2003; Meeks 2003). HRF induces expression of the *N. punctiforme* gene *hrmA* and strains mutated in this gene form hormogonia in the presence of HIF. *hrmA* is part of the *hrmRIUA* operon which resembles sugar uronate metabolism operons of other bacteria, although *hrmA* itself has no sequence homology with any gene in the databases (Campbell et al. 2003; Meeks 2003). The *hrm* locus is central to repressing further hormogonia formation in the *Anthoceros–Nostoc* symbiosis. The gene product of *hrmR* is a transcriptional repressor belonging to the LacI/GalR family of sugar-binding repressors and binds galacturonate in vitro (Campbell et al. 2003). The gene is self-regulating and also regulates *hrmE*, whose function is unknown. Both *hrmR* and *hrmE* are negatively regulated by fructose (Ungerer et al. 2008). Interestingly, immediately downstream of *hrmE* are homologues of genes involved in fructose transport (Ungerer et al. 2008) that are known to be induced by HRF and are therefore considered to be part of the *hrm* locus (Meeks, 2006). Hormogonia formation is known to be repressed by specific sugars, with sucrose being the most effective, followed by glucose and fructose (Khamar et al. 2010). Taken together these observations raise the possibility that fructose (possibly converted to a signalling metabolite) might regulate hormogonia formation (Ungerer et al. 2008). Indeed, the high concentrations of glucose and fructose in *Gunnera manicata* mature stem gland tissue led Khamar et al. (2010) to apply the model of Campbell et al. (2003) and conclude that *hrmR* would be sugar bound and therefore inactive, thus ensuring the expression of genes that negatively regulate hormogonia formation.

In summary, it would appear that free arabinose and galactose present in the *Gunnera* mucilage efficiently attract *Nostoc* hormogonia (Sect. 4; Nilsson et al. 2006) but do not interfere with their formation, allowing them to reach their sites of infection within the host plant. However, inside the *Gunnera* stem gland tissue the high levels of glucose and fructose suppress further hormogonia formation and promote heterocyst differentiation and nitrogen fixation (Khamar et al. 2010).

The *hrm* operon may be involved in symbiotic systems other than *Anthoceros* because expression of *hrmA* in *N. punctiforme* is induced by the plant flavonoid naringin (Cohen and Yamasaki 2000) and by aqueous extracts of fronds of *Azolla pinnata* and *A. filiculoides* (Cohen et al. 2002). Expression of *hrmA* is also induced by deoxyanthocyanin (the pigment that gives *Azolla* its reddish colour in winter) in synergy with other plant-derived compounds (Cohen et al. 2002). The pigment may be a component of *Azolla* HRF as it is more abundant in mature frond tissue than in

actively growing apical regions where hormogonia-like filaments are involved in the infection of newly formed leaf cavities (Cohen et al. 2002).

5.2 Cell Division Control

The cyanobionts of plants have the potential for much more rapid growth than the host and so in symbiosis their growth must be controlled to ensure the stability of the partnership. Although not well understood, this growth control is thought to be achieved by a variety of means such as the blocking of cell division, the restriction of the number and size of symbiotic structures (by, for example, physical confinement) and restriction of the nutrient supply (Rai et al. 2000; Ekman et al. 2006; Bergman et al. 2007a). For example, the *Nostoc* cyanobiont of the hornwort *Anthoceros* grows up to tenfold more slowly in symbiosis than when free living (Meeks 2003). *Anthoceros* also regulates the biomass and rate of nitrogen fixation of its *Nostoc* colonies to maintain a stable rate of nitrogen fixation per unit of plant tissue when, for example, growth is stimulated by elevated CO_2 or light (Meeks and Elhai 2002; Meeks 2003). The mechanism by which this is achieved is not known.

In *Azolla* the growth rates of both host and cyanobiont follow a decreasing linear gradient from their maximum in new leaves at the apical end of the fronds, to a minimum in the older leaves in the mature regions of the plant (Bergman et al. 2007a, b). The host appears to directly regulate cyanobiont growth because when plant growth is artificially reduced with the inhibitor of eukaryotic protein synthesis, cycloheximide, cell division of the cyanobiont in leaves at the plant apex also stops. Cyanobiont cell size and numbers per leaf cavity increase from apical to older regions of the fern (leaf numbers 1–15), after which (leaves 15–28) the numbers per cavity become constant, whereas cell size continues to increase (Bergman et al. 2007a, b).

5.3 Heterocyst Development

Although the presence of heterocysts is essential for a stable nitrogen-fixing symbiosis, mutants unable to differentiate heterocysts may still be able to infect a host plant even though they are unable to support growth of the plant in the absence of combined nitrogen. This is true for mutants of *N. punctiforme* inactivated in *hetR* and *hetF* (Wong and Meeks 2002); the former gene encodes the primary driver of heterocyst development (Golden and Yoon 2003; Zhang et al. 2006), and the gene product of the latter is involved in the regulation and localisation of *hetR* transcription (Wong and Meeks 2001). Although incapable of fixing nitrogen for the symbiosis both mutants infect the hornwort *Anthoceros* as efficiently as the wild type. By contrast, inactivation of the *ntcA* gene, encoding the global nitrogen

regulator NtcA (Flores and Herrero 2005), completely prevents infection even though the mutant makes motile hormogonia (Wong and Meeks 2002).

Plant hosts are capable of supplying fixed carbon for both themselves and the cyanobiont, enabling the latter to increase nitrogen fixation capacity by committing a greater number of CO_2-fixing vegetative cells to become heterocysts (which do not fix CO_2). By adopting a heterotrophic mode of nutrition the cyanobionts can also grow in the roots of cycads and the stem glands of *Gunnera*, where there is little or no light. The cyanobionts in plants therefore generally have heterocyst frequencies greatly elevated above the 5% typical of free-living strains. However, heterocyst frequency is not uniform throughout the plant, being lowest (typically 10–15%) in rapidly growing regions (such as the root tip of cycads), increasing to 60% or more in the oldest symbiotic tissue where multiple adjacent heterocysts are often found, although these are very rare in free-living cyanobacteria. In these regions, some of the heterocysts are likely to be senescent or dead (Meeks and Elhai 2002; Meeks 2003, 2009) and, perhaps as a result of this, the highest nitrogen fixation rates are found at intermediate heterocyst frequencies; this is also the region of the *Gunnera* stem where cyanobiont *hetR* expression is greatest (Wang et al. 2004).

An important question is what regulates heterocyst frequency in planta? There is some evidence that the environmental conditions experienced by the cyanobionts, such as low light and a high concentration of host-derived sugars, are responsible for the elevated heterocyst frequencies. For example, the growth of the *Gunnera* isolate *Nostoc* PCC 9229 in the dark in the presence of fructose results in the formation of double and quadruple heterocysts, which are not seen in the absence of the sugar (Wouters et al. 2000).

In free-living cyanobacteria heterocyst development is triggered by nitrogen starvation, which is thought to be perceived via an increase in the intracellular concentration of 2-oxoglutarate (Muro-Pastor et al. 2001; Vazquez-Bermudez et al. 2002; Zhang et al. 2006) which activates the transcriptional regulator NtcA (Herrero et al. 2004; Flores and Herrero 2005; Muro-Pastor et al. 2005). In turn, NtcA enhances transcription of genes encoding both positive (e.g. HetR and HetF) and negative (e.g. PatN and PatS) regulators of heterocyst development (Meeks and Elhai 2002; Herrero et al. 2004; Zhang et al. 2006; Meeks 2009). However, symbiotically associated cyanobacteria do not show the physiological characteristics of nitrogen limitation (Sect. 6) implying that the signal for heterocyst development in symbiosis is supplied by the host. A similar conclusion can be drawn from observations of the behaviour of a *N. punctiforme* mutant defective in the assimilation of nitrate (Meeks and Elhai 2002; Meeks 2003), which imply that in planta the regulation of nitrogen fixation and heterocyst development is plant mediated and independent of the nitrogen status of the cyanobiont (Meeks 2003, 2009). The identity of this plant signal and its target(s) are unknown but it is likely to act prior to activation of the key heterocyst differentiation gene *hetR* (Zhang et al. 2006) and also prior to *ntcA* (discussed further by Meeks and Elhai 2002; Wong and Meeks 2002; Meeks 2009).

5.4 Host Changes

Morphological changes are apparent in both host and cyanobiont following infection, although the most dramatic changes are found in the latter. Such changes in the host often reflect the need for efficient nutrient exchange and they result in increased physical intimacy of the partners. This is not necessary in *Gunnera* because the intracellular location of the *Nostoc* ensures efficient transfer of nutrients. However, the area of contact between host and cyanobiont is increased in some bryophytes by the production of multicellular filaments that grow from the auricle wall in the liverwort *Blasia* and the wall of the slime cavity in the hornwort *A. punctatus*, and infiltrate the cyanobacterial colony (see: Adams 2002a, b; Adams and Duggan 2008). However, such wall ingrowths are absent in many other hornworts, including *Leiosporoceros* (Villarreal and Renzaglia 2006). In cycads, the area of cyanobiont–host contact is increased by elongation of the cells linking the root inner and outer cortical layers (Costa and Lindblad 2002), creating a space filled with mucilage and tightly packed cyanobacterial filaments and traversed by specially elongated host cells (Vessey et al. 2005).

6 N_2 Fixation and Transfer of Fixed Nitrogen

A significant proportion of the nitrogen fixed by plant cyanobionts is released to the host (Table 1); this varies from 50% in *Azolla* to as much as 80–90% in *Gunnera* and hornworts (Adams 2000; Meeks and Elhai 2002; Adams 2002a, b; Meeks 2003, 2009; Bergman et al. 2007a). In liverworts, hornworts and *Azolla* nitrogen is released in the form of ammonia (Meeks 2003; Bergman et al. 2007a), whereas in *Gunnera* it is ammonia and some asparagine (Bergman 2002; Bergman et al. 2007a), and in cycads it is thought to be glutamine and/or citrulline, depending on the cycad genus (Table 1; Costa and Lindblad 2002; Vessey et al. 2005; Lindblad 2009).

Cyanobacteria assimilate ammonia via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Muro-Pastor et al. 2005; Flores and Herrero 2005) and ammonia release is mostly (with the exception of cycad cyanobionts) a consequence of decreased cyanobiont GS activity resulting from either a reduction in the specific activity of the enzyme or in the amount of protein produced, depending on the host (Table 1). In hornworts, the level of GS protein is similar in free living and symbiotically associated *Nostoc* implying that the observed decrease in overall GS activity in symbiosis is due to a post-translational modification of the protein (Meeks and Elhai 2002; Meeks 2003, 2009).

Because cyanobionts release a large proportion of the nitrogen they fix, they might be expected to show the signs of nitrogen starvation (Meeks and Elhai 2002). In free-living cyanobacteria, this would be evident by degradation of the specialised nitrogen storage compound cyanophycin (a co-polymer of arginine and aspartic

Table 1 Heterocyst frequency and the characteristics of glutamine synthetase in symbiotically associated cyanobacteria

Host	Heterocyst frequency (%)[a]	Glutamine synthetase[b]		Form of combined nitrogen released (% released)[c]
		Amount of protein (%)	Specific activity (%)	
Cycads	17–46	100	100	Glutamine/citrulline? (ND)
Gunnera	20–60	100	70	NH_4^+ (90)
Hornworts and liverworts	25–45	86–100	~15	NH_4^+ (80)
Azolla	26–45	5–40	~30	NH_4^+ (40)
Lichens				
Bipartite	4–8	<10	<10	NH_4^+ (90)
Tripartite	10–55	<10	<10	NH_4^+ (90)

ND not determined

Table compiled from Meeks (2009) and Adams (2000) and references therein

[a]Heterocyst frequency is expressed as a percentage of the sum of heterocysts plus vegetative cells. The range of frequencies reflects the increasing heterocyst frequency found with increasing age of symbiotic tissue. Typical frequencies for free-living cyanobacteria are 4–10%

[b]Glutamine synthetase is expressed as the amount of protein or the specific activity, both expressed as a percentage of the value in the same cyanobacterium growing in the free-living state

[c]The form of combined nitrogen released by the cyanobacterium to the host is given, with (in parenthesis) the amount of nitrogen released by the cyanobiont as a percentage of the total nitrogen fixed

acid) and proteins in carboxysomes (Rubisco) and in phycobilisomes (containing accessory photopigments, the phycobiliproteins). However, all of these reserves are found in relative abundance in symbiotically associated cyanobacteria.

7 CO_2 Assimilation and Transfer of Carbon

Plant cyanobionts receive much of their fixed carbon from the host and their own CO_2 fixation capacity is greatly reduced (Table 2) as they adapt to a photo- or chemo-heterotrophic form of nutrition (Meeks and Elhai 2002; Adams 2002a; Meeks 2003). In the hornwort *Anthoceros,* this seems to be achieved by an unknown post-translational modification of the enzyme Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase, the primary carboxylating enzyme in cyanobacteria), as the amount of the protein differs little between free-living or *Anthoceros*-associated *Nostoc* (Meeks and Elhai 2002; Meeks 2003). However, the mechanism must differ in the cyanobionts of cycads (Rai et al. 2000; Adams 2000; Costa and Lindblad 2002) as Rubisco is active in extracts of freshly isolated cyanobiont (Table 2), despite their heterotrophic mode of nutrition in planta (Lindblad 2009). Photosynthetic CO_2 fixation seems to be reduced in the *Nostoc* symbiont of *Gunnera* by modifications of key photosynthetic components (such as the D1 protein of the photosystem II complex; Black and Osborne 2004). In *Azolla* Rubisco mRNA is 5–7 fold less abundant in the cyanobiont compared with free-

Table 2 Characteristics of light-dependent CO_2 fixation and ribulose bisphosphate carboxylase in symbiotically associated cyanobacteria

Host	Light-dependent CO_2 fixation (%) [a]	Rubisco	
		Protein (%)[a]	Specific activity (%)[a]
Cycads	0	ND	87–100
Gunnera	<2	100	100
Hornwort (*Anthoceros punctatus*)	12	100	12–15
Azolla	85–100	ND	ND
Lichens			
Bipartite	ND	~100	ND
Tripartite	~8	~100	~8

ND not determined
Table compiled from Meeks (2009) and Adams (2000) and references therein
[a]Percentages given are the value in the cyanobiont immediately after isolation from the host, compared with that in the free-living strain

living cultures (Adams 2000; Rai et al. 2000; Meeks 2009), yet immediately after isolation from the plant the primary cyanobiont has approximately 85% of the photosynthetic rate of free-living cyanobacteria (Table 2); why this should be is not known.

8 Artificial Cyanobacteria–Plant Symbioses

The capacity of cyanobacteria for nitrogen fixation has clear potential applications in agriculture for the supply of combined nitrogen to plants, but there are no cyanobacterial endosymbioses with crop plants. Only *Azolla* has been used as a green manure in agriculture, being grown in rice fields, releasing its nitrogen to the soil upon death and decay. However, attempts to induce novel interactions between cyanobacteria and plants have been numerous. Cyanobacteria have been introduced into higher plant protoplasts (see: Rai et al. 2000; Adams 2000; Gusev et al. 2002; Bergman et al. 2007a), or co-cultured with plant tissue cultures, plant regenerates and cuttings from a variety of plants (Gantar 2000b; Gorelova 2001, 2006; Lobakova et al. 2001a, b; Gorelova and Korzhenevskaya 2002; Gorelova and Kleimenov 2003; Gorelova and Baulina 2009; see also Rai et al. 2000 and Gusev et al. 2002 for a discussion of the earlier literature).

Some *Nostoc* hormogonia can be attracted by exudates of non-host plants (Nilsson et al. 2006) and cyanobacteria can also colonise the surface of the roots of rice (Nilsson et al. 2002; 2005) and wheat (Karthikeyan et al. 2007, 2009). Colonisation of wheat seedling roots can enhance plant nitrogen content and root growth (for a discussion of this, see Rai et al. 2000; Adams 2000; Gusev et al. 2002; Bergman et al. 2007a) and cyanobacteria can be induced to grow within root tissues by mechanical damage of the root (Gantar 2000a). Little is known about the extent of such interactions in the field, but the enhancement of such "natural" interactions

may offer the best hope of employing cyanobacteria to enhance crop growth and replace at least a proportion of the current artificial fertiliser use. This might involve, for example, producing crop plants with enhanced release of factors that stimulate hormogonia development and chemoattraction, thus increasing root colonisation. Certainly, this would be a great deal less technically challenging than the creation of novel endosymbiotic associations with crop plants, in which the cyanobiont is retained between host generations. Indeed, in the long evolutionary history of cyanobacteria–plant symbioses *Azolla* is the only such symbiosis to have evolved.

Because most research to date has focussed on the cyanobacteria, our understanding of the plant host involvement in these symbioses is relatively poor. The recent work of Khamar et al. (2010) examining changes in the expression of *Gunnera* genes encoding enzymes for starch and sucrose hydrolysis is the first real attempt to assess changes in host gene expression in response to the symbiotic state. Nevertheless, we still know relatively little about the chemical signals and molecular mechanisms involved in the establishment of a stable cyanobacteria–plant symbiosis.

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Signalling in Ectomycorrhizal Symbiosis

Judith Felten, Francis Martin, and Valérie Legué

Abstract The mechanism by which tree roots and soil fungi interact and form their common, symbiotic organ, the ectomycorrhiza (ECM), involves numerous steps. During this ontogenic process, the developmental programs of both partners are modified in order to enable symbiosis establishment. Both roots and fungus release an array of various metabolites (morphogens and signalling molecules) that establish a molecular cross-talk between symbionts. In contrast to some other plant–microbe interactions, such as rhizobia or arbuscular mycorrhiza symbiosis, the characterization of these signalling molecules and their impact on developmental pathways is poorly known. Recent studies have provided new insights into specific phases and signalling pathways of ECM development on a molecular level and have thereby started to fill the gaps in our understanding of root–fungus communication. Based on this knowledge and recent data from ECM interaction, we will identify possible crosstalk between ECM signalling and root development.

1 Structure and Development of Ectomycorrhizae

While only around 3% of seed-bearing plants establish an ectomycorrhizal (ECM) symbiosis with fungi, ECMs are the most common form of symbiosis for the majority of boreal and temperate forest trees. The fungal community involved in

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ECM formation concerns a great diversity of species among Basidiomycota and Ascomycotina fungi (Smith and Read 2008). Plants species forming ECM belong to Pinophyta and Angiosperms in temporate, boreal, and partially tropical forest (Smith and Read 2008).

The crucial impact of ECM fungi on tree nutrient uptake, including uptake of major nutrients such as nitrogen (N), phosphorus (P), and magnesium (Mg), is well established (reviews Chalot et al 2006; Martin and Nehls 2009). In parallel, the change in tree carbon (C) allocation caused by ECM fungi is considerable. These metabolism activities confer a major role of ECM in ecological and biogeochemical processes in forests (Taylor 2002; Buée et al. 2009).

The functioning structure defined as an ECM is characterized by a basic pattern including the presence of a typical mantle of fungal hyphae around the root, and a labyrinthine inward growth of hyphae between epidermal and (in some species) cortical cells, called the Hartig net (Blasius et al. 1986; Fig. 1a), while the intracellular penetration is scarce. An outwardly growing network of hyphal elements, the extramatrical mycelium, is seasonal present. This well-conserved feature drives the nutrient flow between the two partners (Martin 2007). Extramatrical hyphae gather nutrients (mostly N and P) from the soil and transport them to mantle hyphae, where they may be stored. From there, nutrients are transported to Hartig net hyphae and delivered to and taken up by root epidermis (and cortex) cells. Vice versa,

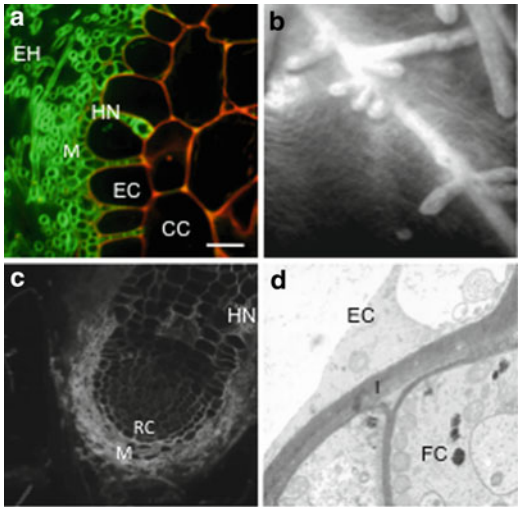

Fig. 1 Anatomy and structure of ectomycorrhizae. (**a**) *Populus tremula x Populus alba*/*Laccaria bicolor* root cross section. Fluorescent double staining showing peripheric root cells (*red cell wall*) and fungal hyphae (*green cell wall*). Mantle (M), Hartig Net (HN), extramatrical hyphae (EH), root epidermis cells (EC), root cortex cells (CC). Bar 10 µm. (**b**) Ectomycorrhizal *Poplar*/*Laccaria bicolor* short roots. (**c**) Poplar root apex colonized by *Laccaria bicolor*. The mantle (M) is present in the root apex, but the Hartig net is developed behind the root meristem. The ECM is accompanied with a reduced root cap (RC) and a small apical meristem zone. Bar 20 µm. (**d**) Interface (I) between root cells (RC) and fungal cells (FC) inside the Hartig net. Courtesy J. Gérard (Nancy University)

photoassimilates (sugars) are released by plant root cells and taken up by the fungus before (partially) being transported back into extramatrical hyphae that use them for further growth and fruiting body development. Together, the different structures and nutrient flow stimulate the growth and health of the plant on several levels: (1) thanks to the wide network of extramatrical hyphae, it benefits from nutrient resources from a greater soil volume than it could exploit by its roots alone (Rousseau et al. 1994), (2) the secreted enzyme activities, which increase mineral solubilization in the soil, can enhance nutrient absorption by plant roots (Landeweert et al. 2001), and (3) the enclosure of the root by the fungus protects plant roots against pathogens but also against inorganic agents. In specific cases, the ability of fungi to absorb and store heavy metals (Blaudez et al. 2000) can even open an ecological niche for the plant that it would not be able to grow in without being associated with ECM fungi.

Like in the formation of endomycorrhizae, ECM development involves a series of complex events. To elucidate such events, most research has employed simplified *in vitro* systems that are conducted to separate sequential phases (Horan et al. 1988; Martin and Tagu 1999; Fig. 2). In the first precontact phase, commonly called early phase, the fungus is attracted towards the root from a soil propagule by increasing hyphal branching and directional growth or from spores by inducing their germination. Fungal hyphae progressively colonize and adhere to the root epidermis (Fig. 2c, d). The docking process that consists of hyphae attachment to root epidermal cells starts with the formation of an "adhesion pad" through aggregation of hyphae (Jacobs et al. 1989). It has been proposed that the initial interaction of hyphae and roots during colonization occurs close to the root tip and that subsequently hyphae invade the root from root cap cells in- and upwards to the epidermis (Horan et al. 1988). Depending on the species, either a mantle of multiple layers of hyphae that multiply and differentiate (Horan et al. 1988) forms first around the root from which the Hartig net develops inward, or occasionally the Hartig net can establish first and the mantle forms afterwards (Nylund and Unestam 1982).

Once colonization of a certain root part is accomplished and the mantle and Hartig net are well developed, the nutrient exchanges take place between both partners in the Hartig net, leading to the functional mycorrhiza, called late phase (Fig. 2e). Furthermore, an equilibrium is established in terms of colonization: depending on plant and fungal species, the Hartig net attains a certain depth into the cell layers of the root (from the epidermis cells to cortex cells) and once colonization is accomplished the fungus does not penetrate any further. The equilibrium (biotrophic) phase, where efficient nutrient exchange occurs, may be maintained for a certain period of time, on the species involved (Smith and Read 2008), until the mycorrhiza ages and undergoes senescence (Dexheimer et al. 1986). It has been shown that ageing mycorrhizas may lose their fungal mantle, even if the Hartig net is maintained, and that their contribution to plant–fungus nutrient exchange diminishes (Al-Abras et al. 1988). The fungus can return back to a saprophytic lifestyle and invade senescent root cells (necrotrophic phase).

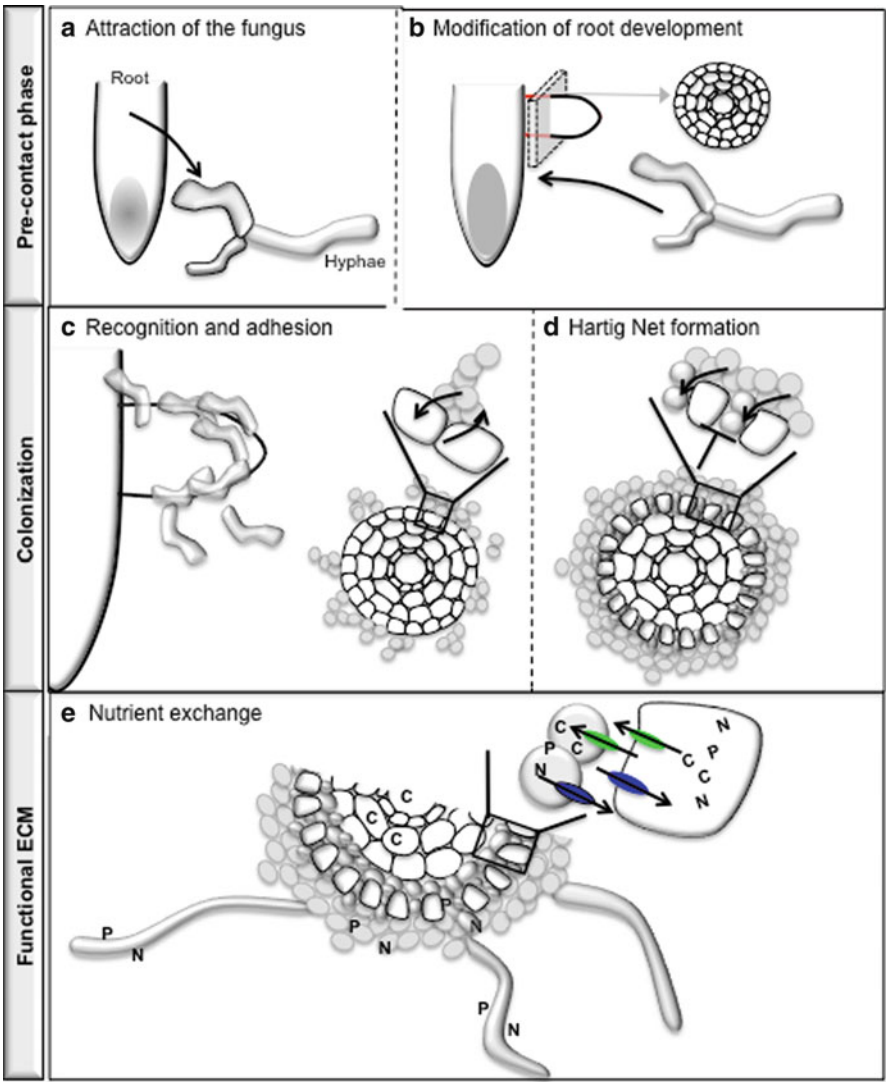

Fig. 2 Scheme of ECM development. ECM development can be separated into three main phases. (1) Signal exchange prior to physical contact, (2) colonization, and (3) nutrient exchange in the functional ECM. The precontact phase is characterized by a plant-to-fungus signalling to induce hyphae branching and attract the fungus (**a**) and to a fungus-to-plant signalling, which stimulates lateral root development of the plant (**b**). The colonization phase may be separated into a first recognition and adhesion of hyphae on the root **(c)** and of penetration of fungal hyphae in between epidermal (and cortex) cells of the plant **(d)**, represented as a transverse root section. The functional ectomycorrhiza is characterized by active nutrient exchange (ammonium (N) and phosphate (P) from the fungus to the plant and sugars (C) from the plant to the fungus). Extramatrical hyphae explore nutrients in the soil and transport them to the mantle and Hartig Net where specific transporters insure the exchange

2 Reprogramming of Root Development

One of the observations when ECM were first described was the presence of numerous short and swollen lateral roots all over the root system (Fig. 1b). The presence of this type of root only during symbiosis illustrates the impact of the fungus on root system development.

The increase in lateral root formation is common for all studied ECM systems. Interestingly, these changes have been observed in response to endomycorrhizae fungi and also in response to bacteria (Olah et al. 2005), suggesting some common traits in the response of the plant host. Moreover, a stimulation of lateral root formation occurs during a change in nutrient availability, including N and P, suggesting a link between the mineral absorption by the fungus during the establishment of ECM and their effects on root architecture.

Recently, it has been observed in an in vitro system that the first visible modification in the induction of lateral roots, which emerge in response to fungal signals, from the parental roots starts in the very early phase of plant–fungus contact, even prior to physical contact between partners (Splivallo et al. 2009; Felten et al. 2009). These observations suggest the importance of a diffusible and/or a volatile signals in this change.

In the more advanced phases of ECM development, another striking change in root development can be observed, i.e., the formation of short roots. The detailed anatomical observations in *Eucalyptus globulus* in contact with Pisolithus clearly reveal a reduced root cap and a small apical meristem zone (Massicote et al. 1987). The differentiation of root tissues including endodermis, stele, and cortex starts closer to the apex. Our observations in poplar–*Laccaria bicolor* follow a broadly similar pattern to that of Eucalyptus (Fig. 1c). In conifer species, dichotomy of the root apical meristem can be observed, which results in short roots that branch just below their tip (Dexheimer and Pargney 1991; Laajanen et al. 2007).

More recently, it has been shown that root growth is guaranteed by the balance between stem cell proliferation and differentiation leading to a specific pattern of root organization. An auxin gradient, dictated mainly by auxin transport and the combinatorial action of some transcription factors, maintains the cell stem niche (Terpstra and Heidstra 2009). Interestingly, the auxin gradient is modified during the first stage of ECM formation (Felten et al. 2009). However, the potential function of transcription factors in the context of ECM is not established and fungal signalling molecules acting on the root apical meristem and columella initials are currently under investigation.

Another striking change in root development is the decay of root hairs in ECM (Ditengou et al. 2000). The absence of root hairs is thought to be due to the physical constraints of the fungal mantle around the root. Root hairs in plants are suggested to fulfill functions in nutrient (N, P) absorption (Bates and Lynch 2000). During symbiosis extramatrical hyphae efficiently take over these functions, so that root hairs are likely to become unimportant for proper ECM root nutrient provisioning.

However, it remains to be elucidated how the root developmental program is altered to halt development of root hairs.

3 Remodelling of Root Cell Walls

Cell walls are important features of plant cells that perform a number of essential functions, including providing shape to the many different cell types. Forming the interface between adjacent cells, plant cell walls often play important roles in intercellular communication. Because of their surface location, plant cell walls also play an important role in plant–microbe interactions.

In the context of ECM, hyphae aggregation and formation of the adhesion pad at the root surface involves recognition between both partners and synthesis of different cell surface structures. The formation of the new interface between the two partners plays an important role in the maintenance and the functioning of the ECM. Ultrastructural studies (Dexheimer and Pargney 1991) reveal that the interface is not formed by the superposition of the fungal wall and the cortical cell walls of the host plant. The parietal structure of the partners is modified to some degree, with, as a result, the production of a polysaccharide-rich cement involved in the cohesion of both partners and reinforcing the anchorage of the fungus to the plant cell surface (Fig. 1d). The presence of chitin has not been detected in this interface. This extensive modification of root cell walls is correlated with the induction of genes encoding cell wall synthesis, loosening and degrading enzymes that may facilitate the entrance of fungi between root cells (Duplessis et al. 2005). The same mycelium can colonize either the epidermal layer only or the cortical layer as well, depending on the host plant. This demonstrates that the host plant controls the fungal growth habit, but the molecular mechanisms are unknown.

Certain fungal cell wall polypeptides are specifically expressed in Pisolithus before or during early phases of Eucalyptus root colonization and are down-regulated later in the mature mycorrhiza (Duplessis et al. 2005; Hilbert et al. 1991; Hilbert and Martin 1988; Martin and Tagu 1995). These are termed “Symbiosis Related Acidic Polypeptides” (SRAPs). Another group of cell wall-related proteins that were overexpressed specifically at the same time points in *P. microcarpus* were hydrophobins. These molecules secreted by the fungus are considered to facilitate the intercellular penetration of hyphae (Nylund 1980) and consequently to have a considerable role in the construction of this new interface and in the communication between the two partners. Hydrophobins are small hydrophobic polypeptides with a conserved distribution of eight cystein residues. Only one class (class I) is found in Basidiomycetes (Wessels 1997). The immunolocalization of one of these hydrophobins, named HYDPt-1, from *Pisolithus tinctorius* (Tagu et al. 2001) reveals its presence in the surface of the hyphae in the mantle and in the Hartig net. The role of hydrophobins in ECM is not well known. It has been proposed that these proteins facilitate the communication between the two partners in participating in the construction of the surface.

In parallel, the establishment of ECM is accompanied by profound modifications in the host cortical cells walls. The orientation of cell growth is radial, suggesting a transformation of the parietal morphogenesis. However, no studies describe in detail the mechanisms involved.

These amazing processes occurring in the host root development implicate a complex signalling network between the two partners.

4 The ECM Fungus Perceives Plant Signals

It has been suggested that root exudates contain compounds that are recognized by fungi and that attract them (Horan and Chilvers 1990). In Eucalyptus, roots release the flavonol rutin that has been identified to stimulate growth of the fungus *P. tinctorius* at only a picomolar in concentration (Lagrange et al. 2001; Martin et al. 2001). It was furthermore shown that the cytokinin zeatin is able to induce branching in ECM hyphae (Martin et al. 2001; Gogala 1991). These factors might be the first communication between a plant and its symbiotic ECM fungus in the early phase (Fig. 2). Their attribution to the early phase can be made because it was shown that when a barrier is present between roots and fungi, compatible fungi will recognize the root and grow towards it (Horan and Chilvers 1990), suggesting that molecules present in root exudates are sufficient for this step. The fact that a flavonol is included in these "branching factors" is an interesting finding, as rhizobia bacteria are also attracted by flavonoids secreted by the plant (Aguilar et al. 1988). This indicates that similarities may occur between different symbioses concerning attraction of the microbial symbiont by the plant partner. Interestingly, the perception of plant exudates not only triggers development of hyphae but also accumulation of metabolites, such as hypaphorine, which may be involved in fungus to plant signalling and will be discussed below (Beguiristain and Lapeyrie 1997). Abietic acid extracted from Pinus roots was able to induce spore germination at a very low concentration (10^{-7} M) and this effect seems to be specific to the genus *Suillus* (Fries et al. 1987). Horan and Chilvers (1990) demonstrated that the presence of root-diffusible molecules is able to chemioattract ECM mycelia.

In the case of AM interactions, studies have revealed the role of strigolactone as a signalling compound to the hyphae aiding in branching and establishment of the mycorrhizae (Gomez-Roldan et al. 2008). Chemical signals released by symbiotic bacteria and by AM fungi are characterized; however, similar molecules have not been identified in ECM.

5 Hormone Signalling During ECM Development

Phytohormones (plant-growth substances) are metabolites that exist in extremely low quantities in plants, or also in microorganisms, and influence in a dose-dependent manner the development of all plant organs. In addition to the five

"classical phytohormones" auxin, ethylene, gibberellins, abscisic acid, and cytokinins (Kende and Zeevaart 1997), today, further molecules such as salicylic acid, brassinosteroids (Grove et al. 1979), and jasmonic acid (Creelman and Mullet 1997) have been assigned as phytohormones and further growth-modifying substances [e.g. strigolactones (Gomez-Roldan et al. 2008; Umehara et al. 2008)] are discussed as having phytohormone character.

In roots, different phytohormones are involved in lateral root development (Fukaki and Tasaka 2009), root meristem maintenance (Benkova and Hejatko 2009), root hair development, and elongation (Rahman et al. 2002; Pitts et al. 1998; Jones et al. 2009) and formation of the root cap (Wang et al. 2005). Auxin is a central element in root development, but, via crosstalk with auxin, other phytohormones also influence root development (Fukaki and Tasaka 2009; Benkova and Hejatko 2009). The fact that during ECM symbiosis root development is altered on all levels suggests that fungal signalling molecules can interact with or alter the root auxin levels that control these patterns. Because of their versatile nature and their production by plants and fungi, phytohormones are interesting candidates to mediate plant–fungus signalling during ECM development.

5.1 Volatile Phytohormones as Precontact Signals

Different studies have suggested that the first fungal signal perceived by the plant does not require any physical contact with the fungus (Splivallo et al. 2009; Felten et al. 2009, 2010). This conclusion has been based on studies that have demonstrated that LR formation in mycorrhizal or nonmycorrhizal plants is stimulated even when ECM truffle fungi or *L. bicolor* are spaced at a certain distance in a Petri dish, or are separated by a semipermeable barrier, from the plant. More strikingly, LR stimulation will even occur when only the exchange of volatiles and not soluble molecules between both partners is allowed (Felten et al. 2010). Therefore, the hypothesis was established that volatile molecules released by the fungus may be the first fungal signal(s) perceived by the plant. ECM fungi are able to produce volatiles such as the phytohormone ethylene (Rupp et al. 1989; Graham and Linderman 1980; Splivallo et al. 2009). Others have demonstrated that the fungus *Fusarium oxysporum* (a nonectomycorrhizal fungus) releases different jasmonates (Miersch et al. 1999). As diffusible molecules such as ethylene and jasmonates are known for their stimulatory effect on LR development (Regvar et al. 1997; Sun et al. 2009; Ivanchenko et al. 2008), they may be some of the key actors in fungal-induced LR stimulation during the precolonization stage. Splivallo et al. (2009) considered ethylene released by ECM truffle fungi as a stimulator of LR development in Arabidopsis as LR stimulation by these fungi was decreased in the ethylene insensitive Arabidopsis line *ein2*. However, in feeding experiments ethylene mimicked fungal LR induction only when applied together with exogenous auxin. Moreover certain ECM fungi, such as *P. tinctorius*, do not produce ethylene but nevertheless stimulate LR development (Rupp et al. 1989). This suggests that

ethylene may be part of the LR stimulating signals exchanged between fungus and plant during the early phase of interaction, but that it is not the only one. Interestingly, the exogenous application of methyljasmonate accelerated the first mycorrhizal contact of spruce roots and *L. laccata* (Regvar et al. 1997), which also suggests a possible role for methyljasmonate during the early phases of ECM establishment. It will be interesting to analyze whether jasmonates are produced by ECM fungi and to further study their effect on ECM development.

It is worth noting that experiments on ethylene levels during colonization have not distinguished between ethylene derived from the plant or from the fungus. The finding of Rupp et al. (1989) that there was an increase in ethylene levels released by roots colonized either with *L. bicolor* (produces ethylene) or with *P. tinctorius* (does not produce ethylene) suggests that the plant partner produces ethylene upon colonization. The same could be valid for jasmonates. Jasmonate and ethylene are usually associated with defense responses against necrotrophic pathogens or herbivore insects (Bari and Jones 2009; Plett 2010), but act as well in biotrophic (mutualistic) associations (Gutjahr and Paszkowski 2009). Consequently, they may be involved in the early defense response during root–ECM fungus contact.

5.2 The Role of Auxin During Early Contact Phase

Auxin, which is one of the major regulators of root development, has been suggested to be at the crosspoint of fungus–plant signalling and the modification of root development during ECM establishment (Felten et al. 2009; Splivallo et al. 2009; Laajanen et al. 2007; Raudaskoski and Salo 2008).

Slankis (1950) was one of the first to suggest a function for auxin released by ECM fungi in the development of ECM root tips. Since then, different research groups have addressed auxin production by ECM fungi in axenic cultures. Techniques ranging from thin-layer chromatography experiments (Ho 1987a, b) to quantification with ELISA techniques or IAA antibodies (Karabaghli-Degron et al. 1998; Rincon et al. 2003) and GC–MS analysis (Splivallo et al. 2009) have all found a very low production of fungal auxin in the nanomolar range (10–300 nM) for different fungi (Ho 1987a, b; Rincon et al. 2001, 2003; Reddy et al. 2006). While results from these studies indicate a large difference in the ability of individual fungal strains to produce IAA (Ek et al 1983; Rudawska and Kieliszewska-Rokicka 1997), even the highest amount of auxin released by certain ECM fungi appears too low to be responsible for early plant responses such as LR stimulation. This is confirmed by the results of Karabaghli-Degron et al. (1998), who demonstrated that LR induction in Norway spruce required 100–500 μM exogenously applied IAA and that below this concentration no effect was observed. However challenging roots with *L. bicolor*, which secretes only about 10 nM IAA, resulted in a visible LR stimulation. Splivallo et al. (2009) came to similar conclusions. They revealed an induction of the auxin response in roots during contact with truffle fungi, as visualized by *Arabidopsis thaliana pDR5: GFP*

lines. However, the fungi only released about 100 nM IAA into the medium, which when exogenously applied did not reproduce the effect of the fungus. Recently, we were able to show that when auxin and *L. bicolor* are simultaneously applied to *A. thaliana*, a cumulative effect is observed concerning LR stimulation, suggesting that fungal signals and auxin can work in synergy to induce LRs (Felten et al. 2010). Taken together these results highlight that fungal auxin in its very low quantity is unlikely to be the trigger of the early plant–fungus signalling. But what about internal root auxin?

We addressed this question in a study where we analyzed whether functional polar auxin transport inside poplar and *A. thaliana* roots was necessary for the fungus to stimulate LR development (Felten et al. 2009). Polar auxin transport occurs due to specific auxin efflux carriers (PIN proteins) situated at distinct sides of the plasma membrane of a cell that mediate the release of auxin from the cell. This directed transport permits auxin to accumulate at specific sites in the root, where it triggers, for example, lateral root primordium initiation or meristem maintenance (Fukaki and Tasaka 2009). Our results showed that polar auxin transport is required for the fungus to stimulate LR formation in poplar or Arabidopsis plants. Interestingly, one specific member of the PIN multigene family, PtPIN9 (orthologue of AtPIN2) was identified as necessary for fungus-induced LR stimulation. Interestingly, this member of the PIN family in Arabidopsis is responsive to jasmonates (Sun et al. 2009). PIN2 induction by the presence of the fungus during the precontact phase is therefore another step towards implication of volatile fungal molecules during the early fungus–plant signalling.

Moreover via their crosstalk with auxin, jasmonates and ethylene are also able to directly influence auxin biosynthesis in plant tissues (Fukaki and Tasaka 2009; Benkova and Hejatko 2009; Stepanova et al. 2007). The observed enhanced auxin response in roots during plant–fungus contact (Splivallo et al. 2009) may therefore be a secondary effect of directed auxin transport or auxin synthesis, not an accumulation of fungal auxin inside plant roots. In conclusion, fungal auxin is likely to play a minor role during the early signalling phase whereas other versatile signals, such as phytohormones ethylene and jasmonates, are likely to indirectly enhance auxin responses inside the root (Fig. 3).

5.3 Auxin as a Possible Signal During Cell Loosening and Hartig Net Development

Low amounts of auxin released by the fungus may be of major importance for root colonization once both partners come into close proximity and released auxin can accumulate inside plant tissues. As part of the "Acid-Growth Theory," auxin, as a weak acid, has been reported to have the ability to loosen the cell wall and to permit growth of cells (Rayle and Cleland 1992). Furthermore, Mensen et al. (1998) proposed that fungal auxin could reduce the peroxidase-catalyzed linkage of the

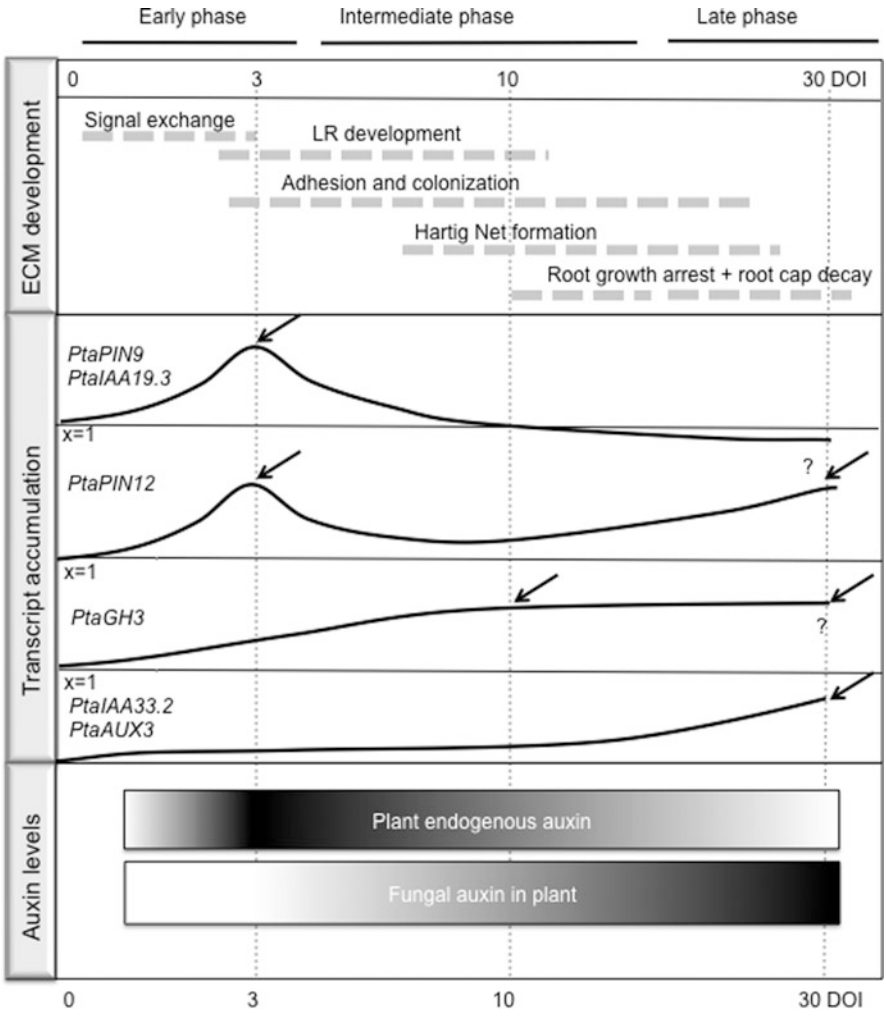

Fig. 3 Auxin homeostasis during ECM development

cell wall constituents, allowing hyphae to penetrate between the cortical cells to form the Hartig net. Results from Tranvan et al. (2000) illustrated nicely how IAA overproduction by the fungus *Hebeloma cylindrosporum* resulted in a quicker and deeper colonization of the root.

Reddy et al. (2006) as well as Charvet-Candela et al. (2002) had reported the differential expression of auxin-inducible genes *PpGH3-16* and *PpIAA88* in *Pinus pinaster* roots during colonization by *H. cylindrosporum* or *Rhizopogon roseolus*. Interestingly, their studies demonstrated no difference in transcript amount between roots challenged with wild type or the IAA-overproducing strain but a faster transcript change when the auxin overproducer was used. This indicates that the

accumulation of fungal auxin inside plant tissues triggers the rapidity and the final outcome of colonization.

In our own study (Felten et al. 2009; Fig. 3) we detected in poplar roots during colonization an accumulation of transcripts related to enzymes (IAA-amido-synthetases GH3) that are able to conjugate auxin to amino acids and thereby mediate dynamically auxin homeostasis (Staswick et al. 2005) and of auxin influx carriers, which import auxin into the cell. It may hence be possible that the plant actively removes fungal auxin from the apoplast and re-adjusts auxin levels. One reason for this could be to divert the free auxin into inactive conjugates or to degrade it. Indeed, Wallender et al. (1992) observed a decrease in auxin levels in mature ECM compared to noncolonized plants. This may be a result of the balance of auxin production by the fungus and auxin degradation by the plant in mature ECM where auxin levels may be drastically reduced to restrict further penetration deeper into plant tissues. Thus, auxin-homeostasis adjustment could be part of the mechanism that regulates the equilibrium of Hartig net colonization (Figs. 3 and 4).

Lastly, Karabaghli-Degron et al. (1998) and Rincon et al. (2001) addressed whether inhibition of polar auxin transport (acting on the plant and apparently not on auxin secretion from the fungus) impacted root colonization. They discovered that the polar auxin transport inhibitor TIBA completely inhibited *Eucalyptus* root colonization by *Pisolithus* and the inhibitor NPA caused an irregular colonization of roots. This finding points to the importance of polar auxin transport inside the plant when fungal auxin accumulates in plant tissues. This transport and redistribution of auxin is likely to be important in enabling colonization.

Also in cases of LRP emergence in the absence of any fungi, distinct auxin maxima that establish due to directed auxin transport have been shown to trigger cell wall remodelling enzymes and to permit cells to separate (Swarup et al. 2008). A similar mechanism may be involved to permit fungal auxin to activate cell separation during Hartig net establishment. Due to the lack of identification of auxin biosynthesis pathways in ECM fungi in contrast to other fungi (Reineke et al. 2008), hitherto no mutants deficient in auxin production are available. Those could confirm our hypothesis that fungal auxin is crucial for root colonization, but does not impact LR stimulation.

Interestingly, an antagonist of auxin has also been shown to be involved in ECM formation. This molecule is the tryptophane-derivative hypaphorine that is produced in large amounts by *P. tinctorius* upon ECM formation (Beguiristain and Lapeyrie 1997). It has been proposed that hypaphorine secreted by the fungus influences root endogenous auxin and thereby regulates symbiosis establishment (Ditengou et al. 2000; Ditengou and Lapeyrie 2000; Jambois et al. 2005). Hypaphorine had been shown to interfere with the actin and microtubular cytoskeleton (Ditengou et al. 2003) and with calcium fluxes (Dauphin et al. 2007), at least in root hairs. However, not all ECM fungi produce hypaphorine. Thus, a general mechanism based on this compound cannot be proposed. Nonetheless, it cannot be ruled out as fungi that do not produce hypaphorine may produce other indole compounds with similar activities.

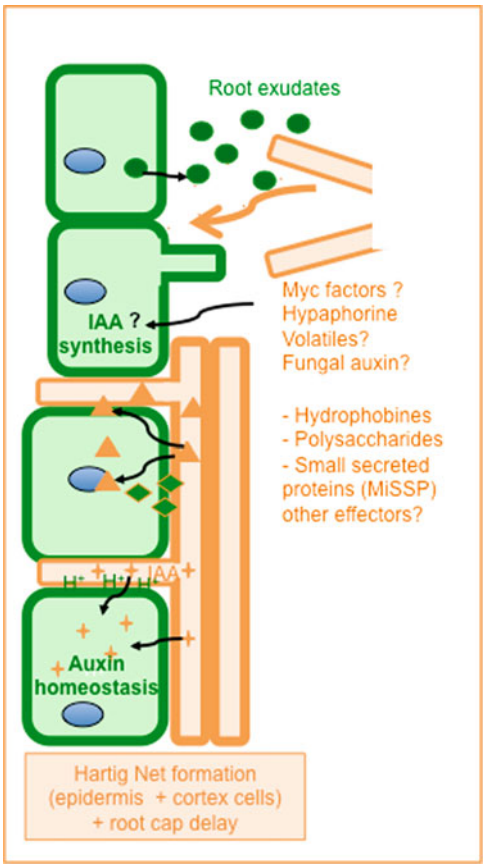

Fig. 4 Signal exchange during the developing ECM. The hypothetical events in the three phases of root colonization are related to root developmental changes observed during poplar root colonization with *L. bicolor*. During the early phase, root exudates induce hyphae branching and attract the fungus. The presence of the fungal signal molecules including volatiles stimulates auxin biosynthesis in plant epidermal cells. This process coincides with LR induction. During the intermediate phase, when colonization takes place, the fungus releases actively IAA through PGP-like carriers into the apoplast of root epidermis cell wall. Together with indole compounds released by the fungus and increased proton effluxes through plasma membrane H^+-ATPases. The induced acidification of the cell wall would facilitate cell spacing and penetration by the fungus. Cells start to decrease excess IAA through conjugation. In parallel, secreted fungal molecules including hydrophobines, polysaccharides and small proteins participate to the Hartig net construction. In the late phase, the fungus still releases IAA into the epidermis cell wall, but plants import this auxin actively and degrade it to prevent further apoplast acification and ongoing penetration of the fungus. Increased auxin degradation may decrease the total auxin content in the cells and also influence root growth arrest and root cap decay that were specifically observed in the late phase. Orange metabolites and proteins are of fungal origin and green ones of plant origin

Taken together, due to its low concentration, fungal auxin is unlikely to be the trigger of LR stimulation in the early phase of root–ECM fungus interaction. Sufficient fungal auxin accumulation in plant tissues is probably only possible

when the contact of both partners is close enough (mantle/Hartig net). In our model (Fig. 3), we propose a gradual change of plant and fungal auxin accumulation at the plant–fungus interface in respective phases of ECM development.

6 Secreted Fungal Molecules as Putative Effectors

Studies developed over the last years have demonstrated that secreted proteins also act as powerful effectors. The activity of these secreted proteins in the manipulation of host-cell structure and function has been broadly reported in many plant–microbial pathosystems (Kamoun 2007). Studies on signalling events in pathogenicity and symbiosis have highlighted a particular class of effectors corresponding to secreted small proteins (SSP). Transcript profiling analysis (Martin et al. 2008) has revealed that these SSPs, so-called Mycorrhiza induced Small Secreted Proteins (MiSSPs), are specially expressed in the Hartig net infection of the ectomycorhizal symbiont, *L. bicolor* in poplar.

Identification of the target molecules is crucial for a deeper understanding of the complex signalling pathways affected in host–symbiont interactions.

7 Evolution of Ectomycorrhizal Signalling

The beginning of ECM fungi evolution is dated to a large time period, probably about 180–130 mya, and it is possible that they arose at different occasions when saprotrophic fungi formed symbiotic partnerships (Hibbett et al. 2000; Alexander 2006; Moyersoen 2006). ECM fungi are therefore younger than endomycorrhizal fungi, which developed 450–350 mya, when plants first settled on land (Simon et al. 1993). The independent evolution of endo- and ectomycorrhizal symbiosis may account for the difference in their signalling mechanisms.

However, parallels can be drawn between ECM signalling and plant interactions with nonbeneficial microbes. Different transcriptome studies have revealed that a defense reaction arises in the plant upon early contact with the fungus, which is repressed at later stages of colonization (Duplessis et al. 2005; Le Quere et al. 2005; Sebastiana et al. 2009). Stress/defense responses involve an accumulation of measurable reactive oxygen species (ROS). ROS production during colonization of roots by ECM fungi is a known phenomenon and is likely to depend on the compatibility of the interacting partners (Gafur et al. 2004). Commonly during microbe–plant interactions an innate immunity response triggers the defense reaction in the plant partner. Innate immunity relies on the perception of a microbe/pathogen associated molecular pattern (MAMP/PAMP) by plant cells via receptors that activate the defense response (Boller and He 2009). But microbes and pathogens can release effectors (polysaccharide, proteins, phytotoxins, etc.) that repress this immune (defense) response. During ECM symbiosis an innate

immunity response has not yet been revealed, but as it is a rather general mechanism, we may suggest that it takes place upon root–ECM fungus interaction. The fact that during ECM symbiosis establishment the stress response is suppressed implies that the fungus secretes such an effector.

8 Conclusions

Even though several studies have addressed the specific signals that trigger ECM symbiosis establishment, the molecules and perception mechanisms involved remain largely unknown. Unlike bacterial or fungal endosymbiosis, where the recognition mechanism (ligand/receptor pair) between plant and fungus and parts of the downstream signalling cascade have been revealed (Stracke et al. 2002; Gherbi et al. 2008; Olah et al. 2005; D'Haeze and Holsters 2002), the early signalling in ECM symbiosis is still a black box. Thus, a clear scheme of factors involved in signalling, recognition, and colonization and their downstream signalling cascade cannot yet be drawn for ECM signalling. However, we propose some hypothetical events in the context of poplar/Laccaria bicolor interaction (Fig. 4). We make an attempt to assign them to specific phases in ECM development. Nonetheless, as data is fragmentary, it is possible that certain molecules are involved in more phases than the ones we have allocated them to in our models.

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Signalling in the *Epichloë festucae*: Perennial Ryegrass Mutualistic Symbiotic Interaction

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Abstract *Epichloë festucae* is a biotrophic fungus that forms symbiotic associations with temperate grasses of the *Festuca* and *Lolium* spp. The association between *E. festucae* and *L. perenne* (perennial ryegrass) is a good experimental system to study these endophyte–grass associations. Integral to the establishment and maintenance of these mutualistic associations is mutual communication between the endophyte and host partner. This communication will likely involve many well-known signalling pathways. Mitogen-activated protein kinase (MAPK) cascades and second messenger signalling pathways involving cAMP and calcium are the main pathways for transduction of signals perceived by cell surface receptors such as G-protein coupled receptors and histidine kinases. Here we review what is currently known about signalling mechanisms in the *E. festucae–L. perenne* symbiosis including a bioinformatics analysis of the *E. festucae* genome to identify which components of these key signalling pathways have been conserved in this organism. Where pathways are yet to be functionally analysed in *E. festucae* we present data from fungal plant pathogens and try to predict possible functions for these pathways in endophyte associations.

1 Introduction

Epichloë endophytes are a group of sexual and asexual clavicipitaceous fungi (Clavicipitaceae, Ascomycota) that form symbiotic associations (symbiota) with temperate grasses of the sub-family Pooideae. There are at least ten different sexual species (Schardl and Wilkinson 2000) including *E. festucae*, a natural symbiont of

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Festuca spp. (Leuchtmann et al. 1994). *E. festucae* also forms a stable mutualistic association with perennial ryegrass, *Lolium perenne*, and is proving to be a good experimental system to study these endophyte–grass associations (Christensen et al. 1997; Scott et al. 2007). The asexual *Neotyphodium* endophytes are predominantly interspecific hybrids that form mutualistic associations with their host (Moon et al. 2004).

These biotrophic fungi systemically colonise the intercellular spaces of leaf primordia, sheaths and blades of vegetative tillers and the inflorescence tissues of reproductive tillers. During the host reproductive phase, sexual species are capable of forming external reproductive structures (stromata) that prevent emergence of the host inflorescence, a disease known as "choke". Initiation of stroma formation appears to be triggered by a switch from restricted symbiotic hyphal growth in the apoplast of the leaf to proliferative pathogenic growth in the outer cell layers and surface of the leaf.

In these mutualistic associations, the major benefits to the fungal symbiont are access to nutrients from the host apoplast and a means of dissemination through the seed. Benefits to the host include increased tolerance to both biotic (e.g. insect and mammalian herbivory) and abiotic stresses (e.g. drought).

2 Biotrophic Lifestyle

Both *N. lolii* and *E. festucae* form stable, asymptomatic mutualistic associations with *L. perenne* and are vertically transmitted through the seed following colonisation of the ovule (Philipson and Christey 1986). After fertilisation, the endophyte colonises the embryo sac and as the seed matures hyphae spreads throughout the embryo (Philipson and Christey 1986; May et al. 2008). During seed germination, hyphae colonise the shoot apical meristem (SAM) from where they enter leaf primordia and axillary buds, the meristematic zones from which new shoots develop. Hyphae appear to grow within the intercellular spaces of the shoot apex by polarised tip growth, and are frequently branched to form a reticulated mycelial network throughout the shoot apex. After colonisation and attachment to the cells within the leaf expansion zone hyphae further elongate by intercalary division and extension (Christensen et al. 2008). This novel pattern of growth allows the fungal endophyte to synchronise its growth with that of the grass host and avoid mechanical shear as the leaf cells are displaced away from the leaf expansion zone. Hyphae grow parallel to the leaf axis but occasionally branch and fuse with adjacent hyphae to establish an extensive intercellular network within the leaf (Fig. 1). At all stages of leaf growth the hyphae are firmly attached to the mesophyll cell walls, providing an ideal connection for nutrient transport and intercellular signalling between host and symbiont (Fig. 2).

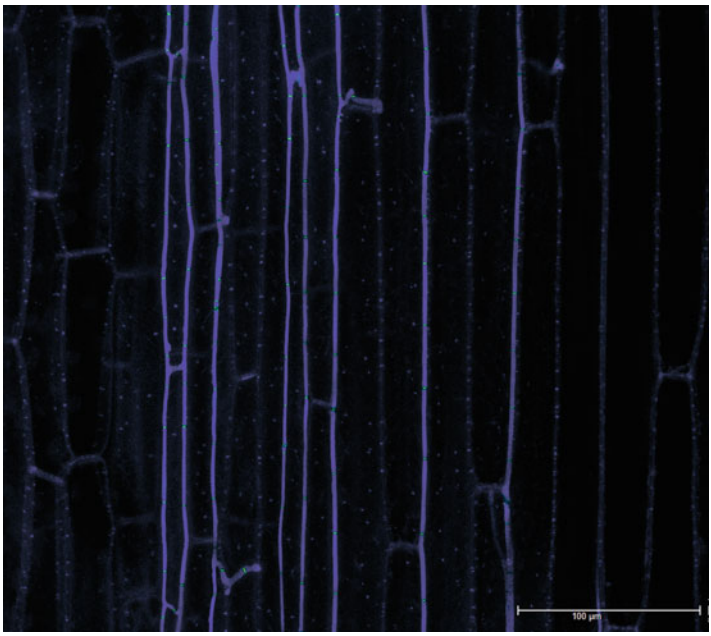

Fig. 1 Symbiotic phenotype of *E. festucae noxR* mutant. Confocal depth series images (1 µm) of longitudinal sections through perennial ryegrass (*Lolium perenne*) leaves infected with wild type (WT) and *noxR* mutant stained with Alexafluor (WGA-AF488) and aniline blue. The image shows hyphae (fluorescent green) of *Epichloë festucae* growing in close association with plant cells. Strongly illuminated points indicate hyphal septa

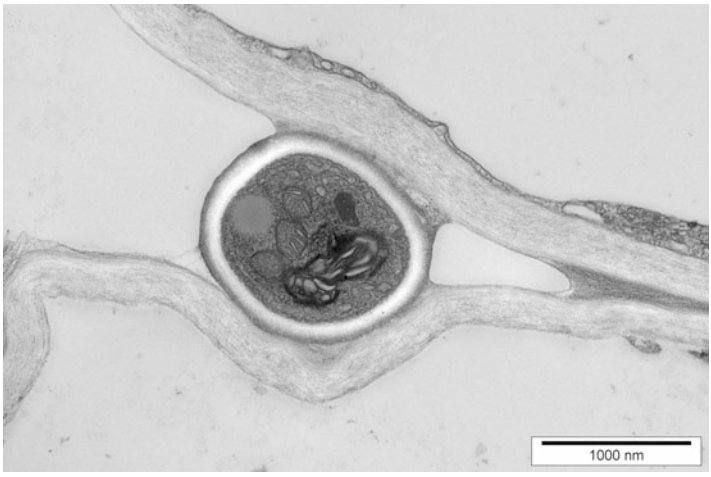

Fig. 2 Transmission electron micrograph showing an *E. festucae* hypha growing in the intercellular space between two perennial ryegrass (*L. perenne*) cells. Bar = 1 µm

3 Regulation of Hyphal Growth in Culture and In Planta

The highly restricted pattern of epichloë endophyte growth in planta implies there must be signalling between the host and the fungus in order to maintain this tightly regulated mutualistic association. Fungal genes involved in host signalling have been identified using a forward genetic screen in which plasmid insertional mutagenesis was used to generate mutants which were screened for any disruption in their association with perennial ryegrass (Tanaka et al. 2006). This strategy identified a mutant that induced severe stunting of leaf growth and resulted in premature senescence of the host. Mutant hyphae were hyperbranched in the leaves and were no longer aligned parallel to the leaf axis. In addition, fungal biomass in planta was significantly greater than in wild-type associations. This dramatic plant interaction phenotype was caused by disruption of *noxA*, a gene that encodes the catalytic subunit of the NADPH oxidase (Nox) complex.

3.1 Regulation of Fungal Growth In Planta by the Nox Complex

The multi-subunit Nox complex is most well characterised in mammalian systems where its role in the respiratory (oxidative) burst response of phagocytes to invading pathogens is well established (Lambeth 2004). The enzymatic function of this complex is to catalyse the conversion of molecular oxygen to the reactive oxygen species (ROS) superoxide, via an NADPH-dependent reaction in which electrons are transferred from intracellular NADPH via FAD and two heme cofactors to molecular oxygen, generating superoxide on the extracellular face of the plasma membrane (Sumimoto et al. 2005).

E. festucae NoxA is most similar to the mammalian Nox2 isoform, known as gp91phox. In mammalian phagocytes, gp91phox is embedded in the plasma membrane where it associates with p22phox to form the flavocytochrome b558 complex, the catalytic core of the oxidase (Groemping and Rittinger 2005). In response to an activating signal, the small GTPase Rac1, and a heterotrimer consisting of p67phox (the Nox activator), p47phox (the Nox organiser) and p40phox translocate to the plasma membrane and associate with gp91phox, leading to the production of superoxide (Groemping and Rittinger 2005). To date only homologues of gp91phox (NoxA and NoxB), p67phox (NoxR) and Rac1 (RacA) have been identified in fungi (Takemoto et al. 2007) Homologues of p40phox and p47phox appear to be absent in fungal genomes, suggesting that fungal specific proteins are required for the recruitment of NoxR and RacA to the plasma membrane (Takemoto et al. 2006). Recent work showed that the yeast polarity proteins Bem1 and Cdc24 interact with NoxR and preferentially localise to actively growing hyphal tips and septa, suggesting they may play an analogous role in fungi to p40phox and p47phox in mammals (Takemoto et al. 2011). Importantly, plants infected with *E. festucae* Δ*noxR*

(Takemoto et al. 2006), Δ*racA* (Tanaka et al. 2008) or Δ*bemA* (Takemoto et al. 2011) mutants also exhibit a defective symbiotic interaction, demonstrating that these additional components of the Nox complex are also essential for symbiotic maintenance. However, it is unclear whether ROS produced by the fungal Nox complex signals to the fungus itself, to the host plant or to both partners. If the fungal Nox complex is localised to the plasma membrane and has the same membrane topology as the phagocytic Nox complex (Sumimoto et al. 2005), ROS would be released into the intercellular space (apoplast) of the host or into the media for cultures grown *ex planta*. Formation of electron dense cerium perhydroxide deposits on the plasma membrane and cell wall of hyphae in planta provides support for the hypothesis that ROS is released into the apoplast (Fig. 3) (Tanaka et al. 2008). This ROS would have the potential to either signal to the plant and/or activate endophyte membrane channels to transduce a signal to the fungal cytoplasm. However, examination of fungal ROS production in culture using nitroblue tetrazolium (NBT), which reacts with superoxide anions to form a dark blue formazan precipitate, revealed the presence of a basipetal ROS gradient within hyphal tips (Tanaka et al. 2006; Semighini and Harris 2008). This suggests that ROS is being generated internally either from Nox located on internal membranes or from alternative enzyme sources (Tanaka et al. 2006; Semighini and Harris 2008; Grissa et al. 2010). Further research is required on the cellular location and topology of fungal ROS producing enzymes to resolve this issue.

3.2 ROS as Signalling Molecules

Given the importance of the Nox complex for symbiotic maintenance, the question arises: how do ROS produced by this complex regulate fungal growth in planta and maintain these associations? ROS are well suited to a role as signalling molecules as they are small and highly diffusible, can be rapidly produced by an NADPH oxidase in response to an activating stimulus and rapidly broken down by antioxidant enzymes (Hancock et al. 2001; Lalucque and Silar 2003). In addition, as Nox

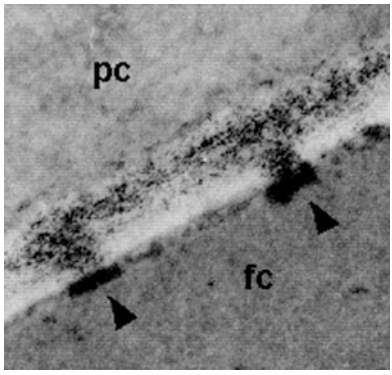

Fig. 3 ROS production by *E. festucae* constitutively active RacA strain in perennial ryegrass. Transmission electron micrograph of H_2O_2 localisation in meristematic tissue. Cerium chloride deposits are indicated by arrowheads. *fc* fungal cell, *pc* plant cell

enzymes are NADPH dependent, their activity is directly linked to cellular metabolism (Lalucque and Silar 2003). However, the main problem with ROS as signalling molecules is their lack of target specificity. As their name suggests, ROS are highly reactive. Hydroxyl radicals, for example, will react with any nearby organic molecule, making them unlikely signalling molecules (Baier et al. 2005). Hydrogen peroxide and superoxide are less reactive so could function as signalling molecules (Baier et al. 2005), although they are unlikely to have specific receptors due to their simple chemical structure (Neill et al. 2002). Instead, it is likely that ROS signal indirectly by oxidation of key signalling proteins, in a reversible process known as redox regulation. Changes in the oxidation state of proteins often lead to changes in their activity (Buchanan and Balmer 2005). The basic mechanism of oxidation of proteins by hydrogen peroxide is well characterised. The hydrogen peroxide molecule reacts with thiolate cysteine residues (RS^-), converting them into sulfenate ions (RSO^-), which rapidly react with nearby thiol cysteine residues (RSH) to form a disulfide bond (Forman et al. 2004). This may lead to conformational changes in the protein that can either reduce or enhance activity, affect cofactor binding or affect binding to other proteins (Allen and Tresini 2000). This oxidation is reversible via antioxidants such as glutathione or thioredoxin, which reduce the oxidised cysteine residue (Lambeth 2004). Two of the best-characterised targets of redox regulation are the transcription factors OxyR (from *E. coli*) and Yap1 (from *Saccharomyces cerevisiae*) (Delaunay et al. 2002; Kim et al. 2002). Reduced OxyR is able to bind DNA, but cannot activate transcription. Oxidation by hydrogen peroxide induces formation of a disulfide bond, which enables OxyR to activate transcription (Kim et al. 2002). In contrast, Yap1 is not directly oxidised by hydrogen peroxide. Instead, a glutathione peroxidase, Gpx3, is oxidised by hydrogen peroxide and forms an intermolecular disulfide bond to Yap1. This bond is then converted to an intramolecular disulfide bond within Yap1, which activates Yap1 and prevents its export from the nucleus (Delaunay et al. 2002). However, it is important to note that the ROS involved in oxidation of these proteins is not necessarily produced by a Nox complex, as this family of enzymes is not found in unicellular organisms such as yeast (Lalucque and Silar 2003). Indeed, most ROS are produced as by-products of aerobic respiration. However, ROS can also be generated enzymatically by some peroxidases (Chen and Schopfer 1999) and potentially by ferric reductases (Takeda et al. 2007; Semighini and Harris 2008).

A range of other enzymatic and non-enzymatic proteins are also subject to redox regulation. These include the protein tyrosine phosphatases, which in contrast to OxyR and Yap1, are strongly inhibited by oxidation (Leslie et al. 2004). The activity of ion channels is also regulated by ROS signalling (Baier et al. 2005). For example, ROS increases outflow of potassium ions through a potassium channel known as human ether-a-gogo (HERG) (Taglialatela et al. 1997), and hydrogen peroxide activates calcium channels in the plasma membrane of *Arabidopsis thaliana* guard cells (Pei et al. 2000). A number of translation initiation and elongation factors, and ribosomal and RNA binding proteins have also been found to be targets of ROS oxidation, providing a link between ROS and translation (Buchanan and Balmer 2005).

ROS molecules are also known to regulate important cellular signalling pathways, and in particular there is a strong link between ROS and calcium (Ca^{2+}) signalling in a variety of organisms. Interactions between Ca^{2+} and ROS signalling systems are very complex and may be both stimulatory and inhibitory, depending on the type of target proteins, the ROS species, dose, duration of exposure and the cell context (Mori and Schroeder 2004; Yan et al. 2006). Many ROS generation processes are calcium dependent. Some NOX enzymes have Ca^{2+} binding regulatory domains and are directly regulated by calcium, as is the case for mammalian NOX5 (Jagnandan et al. 2007), DUOX1 and DUOX2 isoforms (Lambeth 2004). All NOX enzymes in plants are directly regulated by calcium (Torres et al. 1998). In the social amoeba *Dictyostelium discoideum*, signalling of NOXA and NOXB, homologues of gp91phox, is regulated by calcium through Alg-2B, a calcium binding protein, while NOXC has its own calcium binding domains (Lardy et al. 2005). However, deletion of *pef-1*, the *N. crassa* homologue of *alg-2b*, was unable to suppress either the *nox-1* or *nox-2* developmental phenotypes (Cano-Domínguez et al. 2008). NOXC isoforms present in some fungi also contain calcium-binding domains (Takemoto et al. 2007). Calcium signals themselves may be regulated by the redox state of the cell, as is the case for the aforementioned ROS-activated calcium channels of the *A. thaliana* guard cells. ROS also regulates calcium uptake in *A. thaliana* growing root cells. *A. thaliana rhd2* mutants are defective in calcium uptake and their root development is severely impaired, resulting in stunted roots and short root hairs. The *rhd2* gene encodes an NADPH oxidase (Nox) and ROS produced by this protein accumulates in wild-type growing root hairs but are absent from the *rhd2* mutant cells. A direct link between ROS and calcium signalling was established when it was shown that addition of ROS to *rhd2* mutant cells partially suppress the *rhd2* phenotype and stimulates the activity of plasma membrane hyperpolarisation-activated Ca^{2+} channels (Foreman et al. 2003; Takeda et al. 2008). The connection between ROS and Ca^{2+} signalling in *E. festucae* and its importance for the regulation of symbiosis remain to be established.

3.3 Additional Regulators of Fungal Growth In Planta

In addition to components of the fungal Nox complex, two other *E. festucae* genes have also been shown to be essential for symbiotic maintenance. The first of these is the stress-activated MAP kinase, *sakA*, homologous to mammalian p38MAPK or *S. cerevisiae* Hog1. Deletion of *E. festucae sakA* had a dramatic effect on the fungal–host interaction phenotype (Eaton et al. 2010). Infected plants were stunted and senesced within 2–3 months after planting. There was also a dramatic change in plant development, with formation of bulb-like structures at the base of the tillers and almost complete loss of the photoprotective anthocyanin pigments. Fungal growth in planta was deregulated, with hyphal hyperbranching, increased fungal biomass and colonisation of host vascular bundles. Using high-throughput mRNA sequencing to compare fungal and plant gene expression between a wild-type association and the Δ*sakA* mutant association revealed a number of changes

consistent with a transition from restricted hyphal growth in the wild-type mutualistic association to proliferative growth in the mutant association.

Deletion of *E. festucae sidN*, encoding an iron siderophore, also leads to disruption of the *E. festucae*–perennial ryegrass symbiotic interaction (Johnson et al. 2007). The product of SidN is proposed to be a novel ferrichrome-like extracellular siderophore (Johnson 2008). The breakdown of the symbiosis in plants infected with this mutant highlights the importance of iron homeostasis, particularly the ability of the endophyte to access iron from the apoplast, to maintain a stable symbiotic interaction.

3.4 Regulation of Fungal Growth by Calcium Signalling

A central dogma of fungal biology is that hyphae grow by tip growth, a situation analogous to growth of plant pollen tubes and root hairs. Calcium is widely recognised as a crucial molecule in signalling pathways regulating polarised growth. In plants, calcium ions play a crucial role in root hair growth and elongation of pollen tubes. The tip of the growing pollen tube has a cytosolic Ca^{2+} gradient, which is essential for tube growth and is formed by an influx of extracellular Ca^{2+} through stretch-activated ion channels. In the pollen protoplast, these channels are found at the site of tube germination and only after incubation in germination medium (Feijo et al. 2001; Dutta and Robinson 2004).

While tip-localised Ca^{2+} gradients have been observed in polarised fungal hyphal growth there is some debate about the in vivo significance of these gradients because of the lack of reliable techniques for the measurement of cytosolic Ca^{2+} concentrations. Some studies suggest that a Ca^{2+} gradient at the hyphal tip, formed by Ca^{2+} release from the endoplasmic reticulum (ER) in response to activation of stretch-activated phospholipase C and generation of IP3, is essential for hyphal growth (Silverman-Gavrila and Lew 2002, 2003). However, IP3-regulated Ca^{2+} channels are yet to be identified in fungi. Additional studies are needed in order to fully understand the role of Ca^{2+} gradients at the hyphal tip in regulating growth. However, the role of calcium signalling pathways in regulating hyphal growth and branching is well documented (Jackson and Heath 1993; Torralba and Heath 2001). In *N. crassa*, disturbance of the intracellular calcium concentration causes hyperbranching of the growing tip (Schmid and Harold 1988) and application of high concentrations of exogenous calcium restores a wild-type growth pattern to hyperbranched mutants (Kawano and Said 2005). Furthermore, disruption of calcineurin, a Ca^{2+}-regulated phosphatase, induced hyperbranching in *N. crassa*, dissipation of the tip-high Ca^{2+} gradient and eventually growth arrest, suggesting calcineurin is essential for normal tip growth (Prokisch et al. 1997). While nothing is known so far about the importance of Ca^{2+} gradients in the regulation of hyphal growth of *E. festucae*, genes encoding homologues of calcium signalling genes identified in other fungi are found in the *E. festucae* genome (Nguyen et al. 2008) (Fig. 4).

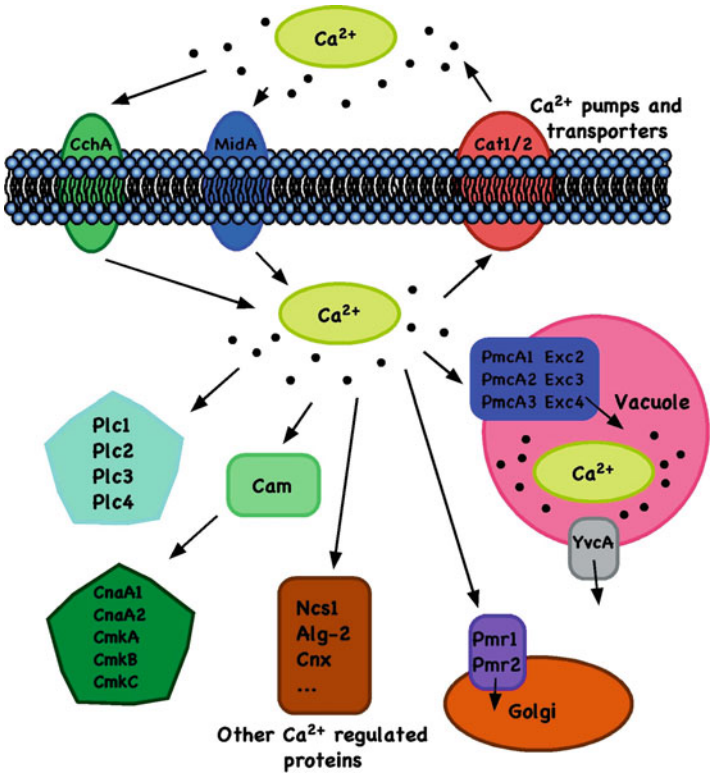

Fig. 4 Proposed components of Ca^{2+} signalling pathways in *E. festucae*. Calcium ions enter the cytoplasm from the extracellular spaces and intracellular depots through a system of Ca^{2+} channels and are actively transported out of the cytoplasm through various Ca^{2+} pumps and exchangers. In the cytoplasm, Ca^{2+} regulates activity of various enzymes like phospholipase C and phosphatases. *E. festucae* contains three Ca^{2+} channels, various Ca^{2+} pumps and transporters, four PLCs, three Ca^{2+}/calmodulin regulated kinases, one or two copies of calcineurin A and various other calcium regulated proteins

4 Signalling Pathways

Undoubtedly, some of the most important signal transduction pathways in filamentous fungi are MAP kinase, heterotrimeric G protein and two-component regulatory pathways. However, with the exception of the stress-activated MAP kinase, these pathways are yet to be studied in epichloë endophytes or any other mutualistic plant symbiotic fungi. This section, therefore, provides an overview of the roles these pathways play in pathogenesis by phytopathogenic fungi. Results of a bioinformatic

survey of the *E. festucae* Fl1 (E894) and E2368 genomes to identify which components of these pathways are encoded in these genomes will also be presented, allowing for predictions as to what roles these pathways may play in endophyte–grass associations.

4.1 MAP Kinase Pathways

Mitogen-activated protein (MAP) kinase pathways are perhaps some of the most conserved and crucial signalling pathways found in eukaryotes. These pathways transduce diverse physical or chemical signals detected at the cell surface into changes in gene expression via a phosphorylation cascade consisting of three highly conserved protein kinases – the MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK). Filamentous fungi generally possess three MAP kinase pathways, which respond to different stimuli – the stress-activated MAP kinase pathway, pheromone response/filamentation pathway and cell wall integrity pathway. The mechanism by which these pathways signal has been extensively reviewed by others (Banuett 1998; Gustin et al. 1998), so will not be covered here.

4.1.1 The Stress-Activated MAP Kinase Pathway

As the name suggests, the stress-activated MAP kinase pathway is activated in response to perception of an environmental stress. The best characterised of these pathways is the Hog1 (*h*igh *o*smolarity *g*lycerol) pathway of *S. cerevisiae*. In *S. cerevisiae*, this pathway is primarily involved in the response to osmotic stress, with mutants in this pathway unable to grow under conditions of high osmolarity (Brewster et al. 1993). In contrast, the homologous Sty1/Spc1 (*s*uppressor of *ty*rosine phosphatase/*s*uppressor of *p*hosphatase 2*C*) pathway of the model fission yeast *Schizosaccharomyces pombe* responds to a variety of different stresses including osmotic, oxidative, temperature and UV stresses (Millar et al. 1995; Shiozaki and Russell 1995). In this regard, the stress-activated MAP kinase pathways of filamentous fungi are more similar to that of *S. pombe*, being able to respond to multiple environmental stresses (reviewed in Rispail et al. 2009).

Interestingly, these pathways in filamentous fungi have been adapted to not only transmit stress signals, but to enhance the virulence of pathogenic fungi. For example, in the dimorphic fungus *Mycosphaerella graminicola*, the causal agent of *Septoria tritici* leaf blotch on wheat, *MgHog1* mutants are non-pathogenic due to an inability to switch to filamentous growth required for infection (Mehrabi et al. 2006). Similarly, *Cochliobolus heterostrophus hog1* mutants have reduced disease symptoms on maize (Igbaria et al. 2008), and *Botrytis cinerea Δbcsak1* MAPK mutants display reduced virulence on bean plants (Segmüller et al. 2007). However,

not all filamentous fungal stress-activated MAP kinases are involved in virulence, as *Magnaporthe grisea* Δ*osm1* mutants (Dixon et al. 1999), *Bipolaris oryzae* Δ*srm1* mutants (Moriwaki et al. 2006) and *Colletotrichum lagenarium osc1* mutants (Kojima et al. 2004) are still fully pathogenic. As discussed in the previous section, signalling through the stress-activated MAP kinase, SakA, is required for maintenance of a mutualistic interaction between *E. festucae* and perennial ryegrass (Eaton et al. 2010). This raises an interesting conundrum – why are stress-activated MAP kinase mutants of some phytopathogenic fungi less virulent, whereas the *E. festucae* Δ*sakA* mutant has increased virulence? It would be interesting to see whether other components of the *E. festucae* stress-activated MAP kinase pathway are also necessary for symbiotic maintenance, or whether regulation of the symbiosis by SakA occurs independently of the MAP kinase pathway. In phytopathogenic fungi, other components of the stress-activated MAP kinase pathway are often also required for virulence, including homologues of the membrane sensors Sho1 and Msb2 (Lanver et al. 2010), the small GTPase Cdc42 (Zheng et al. 2009), p21-activated kinases Cla4 and Ste20 (Smith et al. 2004; Rolke and Tudzynski 2008) and the MAPKKK Ste11 (Zhao et al. 2005). All additional components of the *E. festucae* stress-activated MAP kinase pathway found in the genomes of Fl1 and E2368 are presented in Fig. 5 and Table 1.

4.1.2 The Pheromone Response/Filamentation Pathway

In yeast, the pheromone response/filamentation pathway is primarily involved in perception of a pheromone, which binds to a receptor on the cell surface, activating a heterotrimeric G protein signalling pathway (discussed in more detail later), ultimately leading to activation of the MAP kinase pathway (reviewed in Rispail et al. 2009). The activated Fus3 MAP kinase then targets effectors that induce cell cycle arrest, polarised growth and formation of shmoo cells required for mating (Rispail et al. 2009). Similar to the stress-activated MAP kinase pathway, the pheromone response/filamentation pathway has also been adapted to a role in virulence of phytopathogenic fungi. Indeed, this pathway has been shown to be required for virulence in nearly all phytopathogenic fungi examined, including *M. grisea* (Xu and Hamer 1996), *Fusarium oxysporum* (Di Pietro et al. 2001, 2003), *C. heterostrophus* (Lev et al. 1999), *Alternaria alternata* (Lin et al. 2010), *Botrytis cinerea* (Zheng et al. 2000), *C. lagenarium* (Takano et al. 2000) and *Claviceps purpurea* (Mey et al. 2002). *C. purpurea*, a pathogen of rye, is very closely related to the epichloë endophytes therefore we predict that the pheromone response/filamentation pathway will also play a role in symbiosis between epichloë endophytes and their host plants. Homologues of all major components of the pheromone response/filamentation MAP kinase pathway have been identified in the *E. festucae* Fl1 and E2368 genomes, and are presented in Fig. 6 and Table 1. Interestingly, no obvious homologue of the yeast cyclin-dependent kinase inhibitor Far1 could be identified in the *E. festucae* genomes. Far1 homologues have been identified in other filamentous fungi, including *F. graminearum*, *M. grisea* and

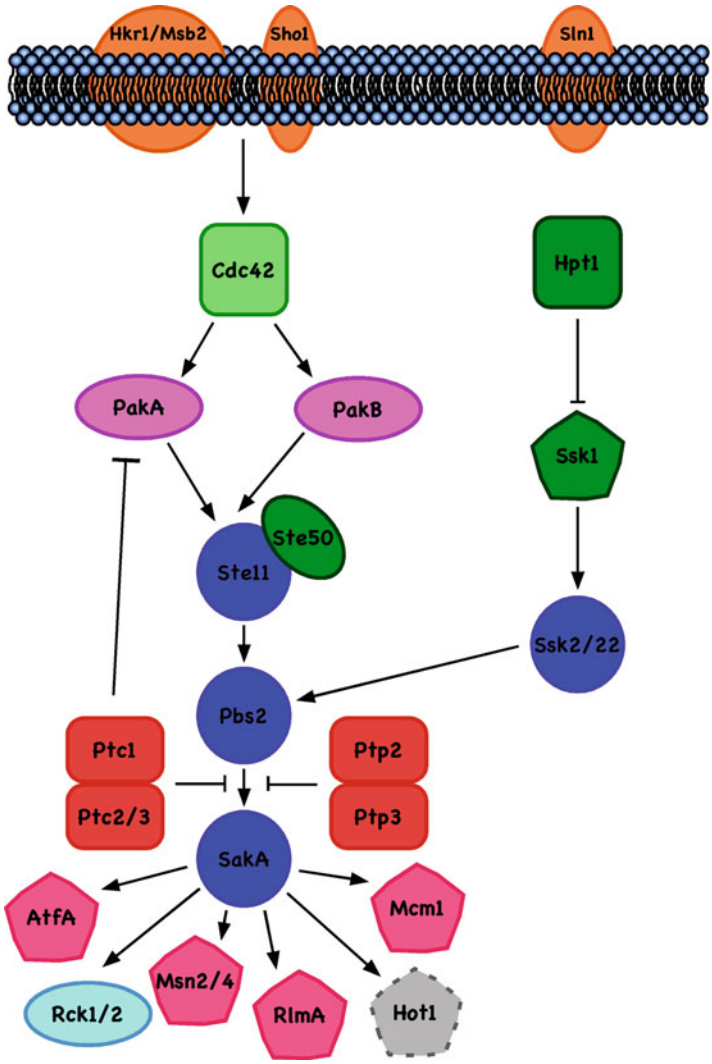

Fig. 5 Proposed components of the stress-activated MAP kinase pathway in *E. festucae*. Schematic showing components of the stress-activated MAP kinase pathway encoded in the genome of *E. festucae* Fl1 and E2368. Proteins are coloured as follows: *orange*, membrane sensor; *red*, phosphatase; *blue*, kinase of the MAPK cascade; *pink*, transcription factor; *light green*, guanine nucleotide exchange factor (GEF); *cyan*, unspecified kinase; *purple*, p21-activated kinase; *dark green*, other function. *Dashed grey boxes* indicate proteins not found encoded in the *E. festucae* genomes. Where proteins have been characterised in other filamentous fungi those gene names are used, otherwise naming is based on *S. cerevisiae* homologues. Adapted from Rispail et al. (2009)

N. crassa (Rispail et al. 2009). The apparent absence of a Far1 homologue suggests that it was either lost in *E. festucae* or it has diverged to such an extent that it is no longer identifiable as a homologue.

Table 1 *E. festucae* genes were identified by tBlastn analysis of the *E. festucae* Fl1 genome (http://lims.ca.uky.edu/CLS894Blast/blast/blast.html) with *M. grisea* protein sequences

E. festucae gene name	*S. cerevisiae* gene name[a]	*M. grisea* locus	*E* value	% identity	Comment
mck1	Bck1	MGG_00883	0	45	MAP kinase kinase kinase
bemA	Bem1	MGG_01702	4e-84	58	SH3-domain protein
mkk1		MGG_06482	0	64	MAP kinase kinase
pkc1		MGG_08689	0	70	Protein kinase C
mps1	Mpk1	MGG_04943	e-177	80	MAP kinase
msg5		MGG_15140	0	52	MAPK phosphatase
pakB	Ste20	MGG_12821	0	59	p21-activated kinase
sakA	Hog1	MGG_01822	e-154	94	MAP kinase
pbs2		MGG_10268	e-166	53	MAP kinase kinase
pmk1	Fus3	MGG_09565	e-179	97	MAP kinase
ptp2		MGG_00912	3e-38	28	Tyrosine protein phosphatase
ptp3		MGG_01376	0	39	Tyrosine protein phosphatase
ssk2/22		MGG_00183	0	60	MAP kinase kinase kinase
ste11		MGG_12855	0	56	MAP kinase kinase kinase
ste50		MGG_05199	e-127	72	Protein kinase regulator
ste7		MGG_00800	e-106	67	MAP kinase kinase
pre1	Ste2	MGG_06452	8e-55	30	Pheromone receptor
pre2	Ste3	MGG_04711	3e-52	34	Pheromone receptor
rgsA	Sst2	MGG_14517	e-140	52	Regulator of G protein signalling
sepA	Bni1	MGG_04061	0	65	Formin
ste12		MGG_12958	0	75	Transcription factor
cdc42		MGG_00466	1e-91	91	Rho GTPase
cdc24		MGG_09697	0	50	Guanine nucleotide exchange factor
gnb1	Ste4	MGG_05201	0	89	Guanine nucleotide-binding protein β subunit
gng1	Ste18	MGG_10193	8e-39	87	Guanine nucleotide-binding protein γ subunit
gna1	Gpa1	MGG_00365	e-160	98	Guanine nucleotide-binding protein α subunit
pakA	Cla4	MGG_06320	0	73	p21-activated kinase
ssk1		MGG_02897	2e-78	40	Cytoplasmic response regulator
hpt1	Ypd1	MGG_07173	9e-28	32	Histidine phosphotransfer protein
sln1		MGG_07312	0	26	Osmosensor histidine kinase
sho1		MGG_09125	e-103	65	Transmembrane osmosensor
msb2		MGG_06033	6e-50	31	Mucin family member
ptc1		MGG_05207	e-157	44	Protein phosphatase 2C homolog
ptc2		MGG_01351	4e-45	61	

(continued)

Table 1 (continued)

E. festucae gene name	*S. cerevisiae* gene name[a]	*M. grisea* locus	*E* value	% identity	Comment
					Protein phosphatase 2C homolog
atfA	Sko1	MGG_08212	e-111	58	bZIP transcription factor
rck1/2		MGG_08547	e-160	68	Serine-threonine protein kinase
msn2/4		MGG_00501	1e-66	41	Zinc finger transcription factor
rlmA	Rlm1	MGG_01204	7e-47	28	MADS-box transcription factor
mcm1		MGG_02773	2e-28	70	MADS-box transcription factor
wsc1		MGG_04325	4e-37	40	Plasma membrane sensor
wsc2/3		MGG_03761	0	42	Plasma membrane sensor
rom1/2		MGG_03064	0	67	Guanine nucleotide exchange factor
tus1		MGG_12644	0	47	Guanine nucleotide exchange factor
sac7		MGG_06390	e-149	41	GTPase activating protein
rho1		MGG_07176	2e-53	90	GTPase activating protein
sit4		MGG_03911	e-107	81	Serine-threonine phosphatase
mbp1/swi4		MGG_08463	e-169	57	DNA-binding component of the SBF complex
swi6		MGG_09869	0	51	Transcription cofactor
ppz1/2		MGG_00149	0	72	Protein phosphatase Z

E values for this analysis and the amino acid sequence identity between the *M. grisea* protein and the predicted polypeptide sequence for the *E. festucae* homologue are given. Proteins chosen for this analysis were based on the MAP kinase pathway schemes presented in Dean et al. (2005) and Rispail et al. (2009). *E. festucae* gene models are available on request[a]Where the *E. festucae* genes are named differently to their *S. cerevisiae* homologues, the *S. cerevisiae* names are also given

4.1.3 The Cell Wall Integrity Pathway

In fungi, the cell wall integrity pathway acts to mediate cell wall remodelling and biogenesis in response to cell wall stress, and during certain stages of the cell cycle (Levin 2005). It does this through a variety of means, including regulation of the glucan synthase and reorganisation of the actin cytoskeleton (Levin 2005). In phytopathogenic fungi, this pathway has also been adapted for a role in virulence. For example, the *C. heterostrophus mps1* MAPK mutant displays reduced virulence (Igbaria et al. 2008), the *C. lagenarium maf1* MAPK mutant is defective in appressorium development and so displays reduced pathogenicity (Kojima et al. 2002), the *M. grisea mps1* mutant is non-pathogenic (Xu et al. 1998), and *F. graminearum* MGV1 MAPK is required for full virulence (Hou et al. 2002). The *C. purpurea* MAPK, CPMK2, is also essential for virulence (Mey et al. 2002),

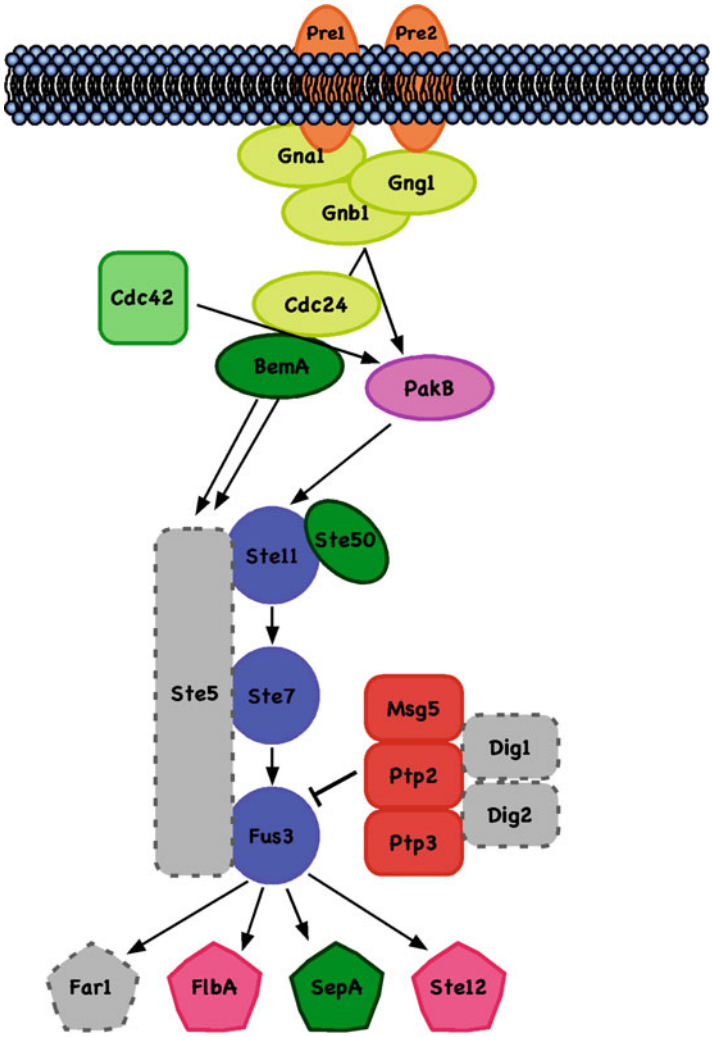

Fig. 6 Proposed components of the pheromone response/filamentation MAP kinase pathway in *E. festucae*. Schematic showing components of the pheromone response/filamentation MAP kinase pathway encoded in the genome of *E. festucae* Fl1 and E2368. Proteins are coloured as follows: *orange*, G-protein coupled receptor; *red*, phosphatase; *blue*, kinase of the MAPK cascade; *pink*, transcription factor; *light green*, guanine nucleotide exchange factor (GEF); *purple*, p21-activated kinase; *yellow*, GTPase; *dark green*, other function. *Dashed grey boxes* indicate proteins not found encoded in the *E. festucae* genomes. Where proteins have been characterised in other filamentous fungi those gene names are used, otherwise naming is based on *S. cerevisiae* homologues. Adapted from Rispail et al. (2009)

and given its close phylogenetic relationship to *E. festucae* we would predict that the cell integrity MAP kinase of *E. festucae* will be required for symbiotic maintenance. It is likely that other components of this pathway will also be required for

symbiosis signalling, given their importance for virulence in phytopathogenic fungi (Jeon et al. 2008). All components of the *E. festucae* Fl1 and E2368 cell integrity MAP kinase pathway have been identified and are presented in Fig. 7 and Table 1.

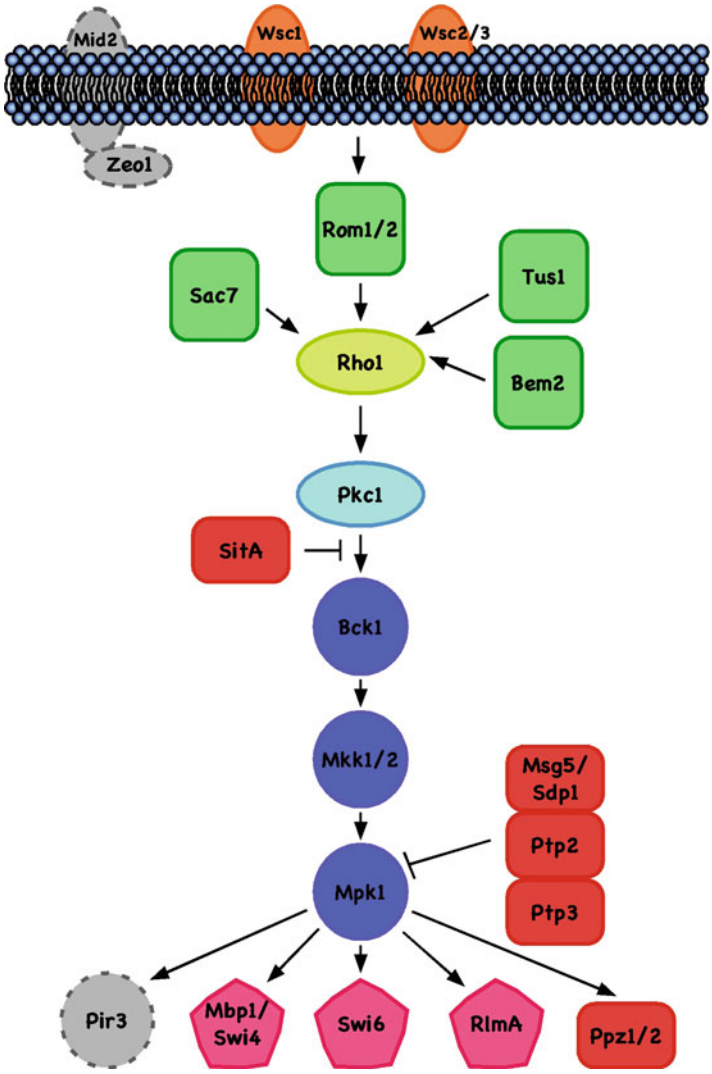

Fig. 7 Proposed components of the cell wall integrity MAP kinase pathway in *E. festucae*. Schematic showing components of the cell wall integrity MAP kinase pathway encoded in the genome of *E. festucae* Fl1 and E2368. Proteins are coloured as follows: *orange*, membrane sensor; *red*, phosphatase; *blue*, kinase of the MAPK cascade; *pink*, transcription factor; *light green*, guanine nucleotide exchange factor (GEF) or GTPase activating protein (GAP); *yellow*, GTPase; *cyan*, unspecified kinase. *Dashed grey boxes* indicate proteins not found encoded in the *E. festucae* genomes. Where proteins have been characterised in other filamentous fungi those gene names are used, otherwise naming is based on *S. cerevisiae* homologues. Adapted from Rispail et al. (2009)

4.2 Heterotrimeric G-Protein Signalling

Filamentous fungal heterotrimeric G proteins were first identified in *N. crassa* (Turner and Borkovich 1993), and much of what we know about G-protein signalling has been determined in this organism (reviewed in Li et al. 2007). In unstimulated conditions, a G-protein coupled receptor (GPCR) on the cell surface is bound to a heterotrimer of Gα (GDP bound), Gβ and Gγ proteins. Binding of an extracellular ligand to the GPCR triggers the GDP on the Gα subunit to be exchanged for GTP. This induces release of the heterotrimer, which then dissociates into GTP-bound Gα and a Gβ/Gγ dimer. These components are then able to interact with downstream effectors. Hydrolysis of the Gα-bound GTP back to GDP induces the Gα to reassociate with the Gβ/Gγ dimer and the GPCR at the membrane (reviewed in Li et al. 2007). The involvement of the heterotrimeric G proteins in virulence of phytopathogenic fungi has been extensively studied. Components of the heterotrimeric G protein signalling pathway encoded in the *E. festucae* Fl1 and E2368 genomes are presented in Table 2.

4.2.1 Gα Subunits

Most filamentous fungi contain three Gα subunits, which tend to play different roles in the cell (Li et al. 2007). In *M. grisea*, only one of these three Gα genes, *magB*, is required for virulence (Liu and Dean 1997). In *C. heterostrophus* only *CGA1* is

Table 2 *E. festucae* genes were identified by tBlastn analysis of the *E. festucae* Fl1 genome (http://lims.ca.uky.edu/CLS894Blast/blast/blast.html) with *N. crassa* protein sequences

E. festucae gene name	*N. crassa* locus	*E* value	% identity	Comment
gna1	NCU06493	e-157	97	Guanine nucleotide-binding protein α subunit
gna2	NCU06729	1e-98	77	Guanine nucleotide-binding protein α subunit
gna3	NCU05206	1e-75	76	Guanine nucleotide-binding protein α subunit
gnb1	NCU00440	e-165	85	Guanine nucleotide-binding protein β subunit
gng1	NCU00041	2e-24	90	Guanine nucleotide-binding protein γ subunit
orp1	NCU01735	2e-38	32	G-protein coupled receptor
pre1	NCU00138	1e-50	20	G-protein coupled receptor
pre2	NCU05758	8e-41	25	G-protein coupled receptor
gpr1a	NCU00786	6e-53	22	G-protein coupled receptor
gpr1b	NCU00786	3e-9	27	G-protein coupled receptor
gpr2	NCU04626	5e-93	40	G-protein coupled receptor
gpr4	NCU06312	6e-38	24	G-protein coupled receptor
gpr5	NCU00300	1e-41	34	G-protein coupled receptor
gpr6	NCU09195	4e-49	51	G-protein coupled receptor

E values for this analysis and the amino acid sequence identity between the *N. crassa* protein and the predicted polypeptide sequence for the *E. festucae* homologue are given. Proteins chosen for this analysis were based on those present in the *N. crassa* genome, as presented in Li et al. (2007). As this analysis focused on identification of homologues of the *N. crassa* G protein signalling proteins, there may be additional G-protein coupled receptors encoded in the *E. festucae* genome that were not identified by this analysis. *E. festucae* gene models are available on request

required for normal appressorium formation (Horwitz et al. 1999), and in *C. parasitica* only *cpg-1* is required for virulence (Gao and Nuss 1996). In contrast, both *Gpa1* and *Gpa3* of *Mycosphaerella graminicola* are required for pathogenicity, while *Gpa2* is dispensable (Mehrabi et al. 2009). Similarly, both *bcg1* and *bcg2* of *B. cinerea* are required for normal virulence (Gronover et al. 2001). Both *Fusarium oxysporum fga1* (Jain et al. 2002) and *Stagonospora nodorum gna1* (Solomon et al. 2004) are required for pathogenicity, but it is still unclear whether the other Gα genes from these fungi are also involved in pathogenicity. Interestingly, *Ustilago maydis* has four Gα genes, but only one of these, *gpa3*, is required for pathogenesis (Regenfelder et al. 1997). Similar to most other filamentous fungi, the *E. festucae* genomes encode three Gα proteins (Table 2). Given the key role these gene products play in phytopathogenesis, it is very likely that at least one of these proteins will be required for symbiotic maintenance.

4.2.2 Gβ and Gγ Subunits

In contrast to the multiple Gα genes possessed by most filamentous fungi, generally only one gene for Gβ and one for Gγ are present in fungal genomes (Li et al. 2007). The Gβ gene product, in particular, has a key role in phytopathogenicity. For example, *C. heterostrophus CGB1* is required for normal virulence on maize (Ganem et al. 2004), and *F. oxysporum fgb1* is required for pathogenicity on cucumber (Jain et al. 2003). In addition, *M. grisea mgb1* mutants are non-pathogenic (Nishimura et al. 2003), and *M. graminicola* (Mehrabi et al. 2009) and *C. parasitica* (Kasahara and Nuss 1997) Gβ mutants have reduced virulence. It is unclear yet what role the Gγ subunit may play in virulence of phytopathogenic fungi. The *E. festucae* Fl1 and E2368 genomes encode products for one Gβ and one Gγ, which are highly conserved with homologues from other fungi (Table 2). We predict that the *E. festucae* Gβ subunit will be crucial for symbiotic maintenance.

4.2.3 G-Protein Coupled Receptors (GPCRs)

GPCRs are the membrane bound component of the G-protein signalling pathway that respond to changes in the environment or ligand binding and transmit the response to the heterotrimeric G proteins. In *N. crassa,* these proteins have diverse functions including pheromone response, cAMP response and carbon sensing (reviewed in Li et al. 2007). At present there are no reports that we are aware of on the role of GPCRs in virulence of phytopathogenic fungi. We predict that the products of at least some of these genes will be crucial for transmitting metabolic signals from the host to the symbiont. The *E. festucae* genomes appear to contain genes for at least nine GPCRs, as identified by their homology to the ten GPCRs originally identified in *N. crassa* (which is now known to encode considerably more GPCRs than this) (Table 2). Interestingly, the *E. festucae* genomes do not appear to encode a homologue of the *N. crassa* opsin NOP-1. In addition, no homologue of

N. crassa GPR-3 could be identified, but two genes showing high homology to GPR-1 were identified.

4.3 Two-Component Regulatory Signalling

Two-component regulatory system (TCS) pathways comprise a membrane-bound histidine kinase and a cytosolic response regulator which, in response to signal perception by the histidine kinase is autophosphorylated, resulting in activation of downstream effectors. The importance of these pathways in environmental sensing by prokaryotes is well established (reviewed in Chang and Stewart 1998). A fungal TCS pathway was first identified in yeast, and its role upstream of the HOG1 stress-activated MAP kinase pathway is now well established (reviewed in Saito and Tatebayashi 2004). In filamentous fungi, a TCS was first characterised in *N. crassa*, following the identification of the two-component histidine kinase, NIK-1 (OS-1) (Alex et al. 1996). With the sequencing of the *N. crassa* genome, ten additional HKs were identified (Galagan et al. 2003). Interestingly, *N. crassa* contains only two response regulators (RRs), RRG-1 (Jones et al. 2007) and RRG-2 (Banno et al. 2007), suggesting that signals from all 11 HKs must be channelled through the two RRs. By comparison to most prokaryotic TCSs, fungal TCSs are generally more complex with the HK being comprised of both a histidine kinase domain and a response regulator receiver domain. Hence, they are referred to as hybrid histidine kinases (HHKs). The histidine kinase domain is autophosphorylated in response to an activating signal and the phosphate transferred to the RR receiver domain within the HHK. In another difference to most prokaryotic systems, the phosphate on the HHK is first transferred to a histidine phosphotransfer protein (HPT) protein, which in turn transfers the phosphate to the RR, resulting in activation of downstream effectors (Stock et al. 2000; Banno et al. 2007).

4.3.1 Hybrid Histidine Kinase

In phytopathogenic fungi, TCS pathways have been adapted for a role in virulence. The HHKs BOS1 from *B. cinerea* (Viaud et al. 2006), Fhk1 from *F. oxysporum* (Rispail and Di Pietro 2010) and CpHK2 from *C. purpurea* (Nathues et al. 2007) have all been shown to be necessary for normal virulence. In contrast, *M. grisea* Hik1 is not required for pathogenicity (Motoyama et al. 2005). The genomes of *E. festucae* Fl1 and E2368 contain at least nine HHKs, based on a comparison of the 11 genes in *N. crassa* (Fig. 8 and Table 3). Interestingly, only one phytochrome is encoded by the *E. festucae* genome compared to the two, PHY-1 and PHY-2, encoded by the *N. crassa* genome.

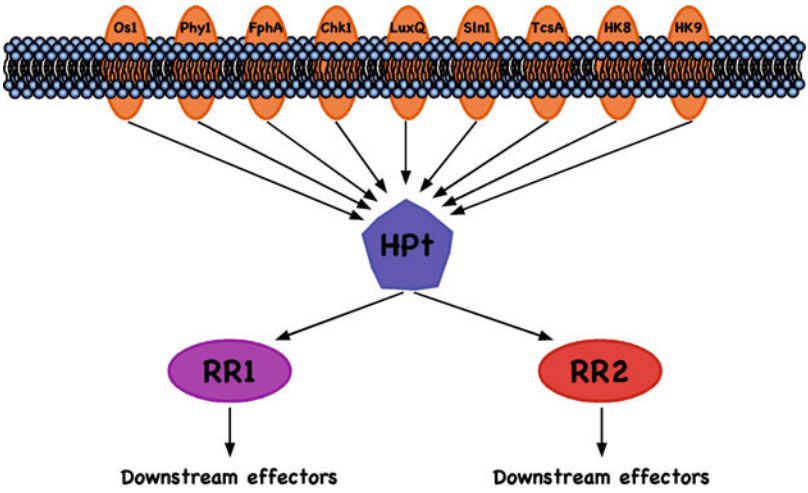

Fig. 8 Proposed components of two component signalling pathways in *E. festucae*. Schematic showing components of two component signalling pathways encoded in the genome of *E. festucae* Fl1 and E2368. Hybrid histidine kinases (HHKs) are represented in *orange*. HHKs were identified based on homology to the 11 HHKs encoded in the *N. crassa* genome. Additional novel HHKs may also be encoded in the *E. festucae* genomes. *HPt* histidine phosphotransferase, *RR* response regulator

Table 3 *E. festucae* genes were identified by tBlastn analysis of the *E. festucae* Fl1 genome (http://lims.ca.uky.edu/CLS894Blast/blast/blast.html) with *N. crassa* protein sequences

E. festucae gene name	*N. crassa* locus	E value	% identity	Comment
rrgA	NCU01895	e-142	33	Response regulator
rrgB	NCU02413	1e-75	40	Response regulator
hpt1	NCU01489	3e-24	39	Histidine phosphotransfer protein
os1	NCU02815	0	79	Histidine kinase
phy1	NCU04834	0	41	Histidine kinase
tcsA	NCU00939	0	45	Histidine kinase
fphA	NCU01823	0	56	Histidine kinase
chk1	NCU01833	0	53	Histidine kinase
luxQ	NCU02057	e-168	49	Histidine kinase
sln1	NCU04615	0	27	Histidine kinase
hhk8	NCU00939	2e-99	39	Histidine kinase
hhk9	NCU09520	e-100	22	Histidine kinase

E values for this analysis and the amino acid sequence identity between the *N. crassa* protein and the predicted polypeptide sequence for the *E. festucae* homologue are given. Proteins chosen for this analysis were based on those present in the *N. crassa* genome (Galagan et al. 2003). As this analysis focused on identification of homologues of the *N. crassa* two-component signalling proteins, there may be additional histidine kinases encoded in the *E. festucae* genome that were not identified by this analysis. *E. festucae* gene models are available on request

4.3.2 Response Regulator and Histidine Phosphotransfer Protein

Very little information is available on the role of fungal response regulators in phytopathogenesis. However, in *Magnaporthe oryzae* one RR (SSK1) and one RR-like gene (RIM15) were shown to be required for virulence (Motoyama et al. 2008). In addition, in *C. heterostrophus SSK1* but not *SKN7* is required for virulence, and in *Gibberella zeae*, *ssk1* mutants show reduced pathogenicity (Oide et al. 2010). The genomes of *E. festucae* Fl1 and E2368 encode two response regulators, homologous to *N. crassa* genes for RRG-1 and RRG-2 (Fig. 8 and Table 3). Given the precedence for a role of RRs in virulence of the phytopathogenic fungi it is likely that at least one of the two *E. festucae* RRs will be required for symbiotic maintenance. To date there are no reports of a role for the HPt in phytopathogenesis, possibly because mutants of this gene are lethal as found for *A. nidulans* (Vargas-Perez et al. 2007). *E. festucae* contains one HPt gene, and like in *A. nidulans* it is predicted to be essential.

4.4 Second Messenger Signalling Pathways

In order to sense and respond to internal and external environmental signals, cells have developed signalling systems that transmit signals from cell surface receptors to their intracellular targets using second messengers. Second messengers are small molecules whose cytoplasmic concentration is controlled in response to extracellular/intracellular signals. This section reviews two of the most important fungal second messengers: cyclic adenosine monophosphate (cAMP) and calcium.

4.4.1 cAMP Signalling

cAMP regulates a wide range of developmental, metabolic and pathogenic processes in fungi. However, despite regulating a diverse range of processes, components of cAMP signalling are highly conserved (D'Souza and Heitman 2001). Synthesis of cAMP is catalysed by adenylate cyclase and its degradation is facilitated by phosphodiesterases. The activity of these enzymes is regulated by various extracellular signals. Adenylate cyclase is directly regulated by heterotrimeric G-protein signalling through protein kinase A (PKA), a key enzyme involved in most cAMP regulated processes. In its inactive state, PKA is a tetramer composed of two regulatory and two catalytic subunits. When the cytoplasmic concentration of cAMP increases, cAMP binds to the regulatory subunits causing disassociation of the catalytic subunits. The two catalytic subunits act downstream on various transcription factors and metabolic enzymes.

In filamentous fungi, cAMP has a key role in the regulation of growth and developmental processes. Inactivation of the PKA regulatory subunit disrupts polarised growth in *N. crassa* (Bruno et al. 1996) and *Aspergillus niger* (Saudohar et al. 2002). cAMP is also important for regulating plant infection by fungal

pathogens, likely due to a role in regulating morphological switching which is often required for pathogenesis. For example, in *U. maydis* cAMP is required for formation of the dikaryon and the subsequent morphological switch to filamentous growth required for infection (reviewed in Kahmann et al. 1999). Mutations in adenylate cyclase, *uac1*, or the catalytic subunit of PKA, *adr1*, induce filamentous growth of haploid cells while mutation in the PKA regulatory subunit, *ubc1*, as expected, has the opposite effect and results in a multiple budding phenotype (Gold et al. 1994; Durrenberger et al. 1998). Both *adr1* and *ubc1* are also involved in the second stage of the infection process, with *adr1* mutants being non-pathogenic and *ubc1* mutants able to penetrate the host but unable to induce tumours. These results suggest cAMP is required for the penetration process but inhibits later steps in infection, such as tumour development. In the maize pathogen *Fusarium verticillioides,* disruption of the PKA catalytic subunit *CPK1* or adenylate cyclase *FAC1* leads to defects in radial growth, but only *fac1* mutants show significantly reduced virulence, suggesting there may be another copy of the catalytic subunit gene which regulates the infection process (Choi and Xu 2010). In *M. graminicola*, disruption of either the regulatory or catalytic PKA subunit significantly reduces virulence (Mehrabi and Kema 2006). Disruption of adenylate cyclase in *B. cinerea* disrupts sporulation and reduces the rate of lesion development during the infection process (Klimpel et al. 2002). cAMP signalling has diverse roles in *M. grisea*. Deletion of the PKA catalytic subunit, *CPKA*, dramatically reduces appressorium formation (Mitchell and Dean 1995). Deletion of the adenylate cyclase gene, *MAC1*, also reduces appressorium formation but it also affects vegetative growth, conidiation and conidial germination (Choi and Dean 1997). Mutations in the PKA regulatory subunit (*SUM1*) suppressed the growth and development phenotypes of *mac1*, including appressorium formation (Adachi and Hamer 1998). Under non-inducing conditions *SUM1* mutants undergo precocious development of appressoria, suggesting CPKA is not the only catalytic subunit present in *M. grisea*. Homologues of adenylate cyclase and the PKA catalytic and regulatory subunits are found in the genomes of *E. festucae* Fl1 and E2368. Disruption of *acyA* (adenylate cyclase) in *E. festucae* reduced radial growth and increased conidiation (Voisey et al. 2007). The most pronounced effects in planta were a reduction in infection rate and an increase in hyphal branching, although host plants did not become stunted as was observed for *noxA* and *racA* mutants.

4.4.2 Ca^{2+} as a Second Messenger

Calcium is a universal secondary messenger, involved in a broad range of processes from gene expression to apoptosis (Bootman et al. 2001), and is proposed to be the most versatile biological messenger known (Cheng and Lederer 2008). Cytoplasmic calcium concentration is maintained at a very low level (100–500 nM) compared with that in the extracellular spaces and internal stores (1–5 mM) (Halachmi and Eilam 1989; Permyakov and Kretsinger 2009). A calcium signal is generated when the cytoplasmic Ca^{2+} concentration increases, which in turn activates Ca^{2+}-regulated

effectors. Calcium ions are transported from the cytoplasm by calcium pumps and ion exchangers.

Calcium Signalling in Fungi

Fungal calcium signalling has been best characterised in the budding yeast *S. cerevisiae*. While relatively little is known about calcium signalling in filamentous fungi, it appears to be more complex than in *S. cerevisiae* (Zelter et al. 2004). This section will provide an overview of the main components of fungal Ca^{2+} signalling pathways, including a bioinformatic survey of calcium signalling components present in the *E. festucae* genome (Table 4 and Fig. 4). Calcium signalling proteins are highly conserved across the fungal kingdom, but with some variation in copy number. While few of the identified calcium signalling genes have been functionally characterised in *E. festucae*, systematic functional analysis of Ca^{2+} genes in the phytopathogenic fungus *M. oryzae* using RNA silencing provides an insight into the importance of these pathways for plant–fungal interactions (Nguyen et al. 2008).

Calcium Channels and Transporters

Three Ca^{2+} permeable channels have been characterised in *S. cerevisiae*: the voltage-gated channel Cch1p, stretch-activated Mid1p channel and the transient receptor potential-like Ca^{2+} channel Yvc1p. Disruption of *mid1* induces loss of cell viability after pheromone-induced cell differentiation, with the mutant displaying low Ca^{2+} uptake. This loss of viability can be restored by incubation in a high extracellular Ca^{2+} concentration medium (Iida et al. 1994). Δ*cch1* mutants appear identical to Δ*mid1* mutants, suggesting Cch1p and Mid1p are components of the same Ca^{2+} permeable channel (Paidhungat and Garrett 1997). The Yvc1p channel is located in the vacuolar membrane (Palmer et al. 2001), and is involved in the calcium response triggered by hyperosmotic shock (Denis and Cyert 2002; Zhou et al. 2003). Knock-down of *M. oryzae* Cch1p leads to reduced sporulation and appressorium formation but the strains remain pathogenic (Nguyen et al. 2008). Knock-down of Mid1p resulted in a similar phenotype, whereas knock-down of Yvc1p strongly affected conidia and appressoria formation (Nguyen et al. 2008). Interestingly, deletion of *C. purpurea mid1* resulted in a complete loss of pathogenicity and production of appressoria-like structures not normally seen in *C. purpurea* (Bormann and Tudzynski 2009). Like other filamentous fungi, *E. festucae* contains homologues of the Ca^{2+} channel proteins Cch1, Mid1 and Yvc1 (Table 4). Given the close relationship between *E. festucae* and *C. purpurea* it is likely that *E. festucae mid1* may be involved in regulating symbiosis.

The role of Ca^{2+} ATPases (Ca^{2+} pumps) and exchangers is to maintain low cytoplasmic Ca^{2+} concentrations by actively removing Ca^{2+} from the cytoplasm. In fungi, these proteins work together with the phosphatase calcineurin in regulating

Table 4 *E. festucae* genes were identified by tBlastn analysis of the *E. festucae* Fl1 genome (http://lims.ca.uky.edu/CLS894Blast/blast/blast.html) with *M. oryzae* protein sequences

Gene name	*M. oryzae* locus	E value	% Identity	Comment
cch1	MGG_5643	0	52	Ca^{2+} channel
mid1	MGG_12128	0	29	Ca^{2+} channel
yvc1	MGG_9228	0	59	Ca^{2+} channel
cnaA1	MGG_07456	0	85	Calcineurin catalytic subunit; present in Fl1 and E2368
cnaA2	MGG_07456	0	62	Calcineurin catalytic subunit; present in E2368, absent from Fl1
cnaB	MGG_06933	0	64	Calcineurin regulatory subunit
Cam	MGG_06884	0	100	Calmodulin
ncs1	MGG_01550	0	83	Frequenin
alg-2	MGG_04818	0	78	Penta-EF-hand Ca^{2+} binding protein
cmkA	MGG_09912	0	65	Ca^{2+}/Calmodulin-dependent protein kinase
cmkB	MGG_00925	0	73	Ca^{2+}/Calmodulin-dependent protein kinase
cmkC	MGG_06421	0	43	Ca^{2+}/Calmodulin-dependent protein kinase
plc1	MGG_02444	0	44	Phospholipase C
plc2	MGG_00211	0	54	Phospholipase C
plc3	MGG_15018	0	34	Phospholipase C
plc4	MGG_08315	0	42	Phospholipase C
	MGG_05332	0	46	
pmcA1	MGG_02487	0	59	Calcium-transporting ATPase
pmcA2	MGG_07971	0	49	Calcium-transporting ATPase
pmcA3	MGG_04890	0	49	Calcium-transporting ATPase
pmr1	MGG_11727	0	70	Secretory pathway Ca^{2+}-ATPase
pmr2	MGG_04550	0	81	Secretory pathway Ca^{2+}-ATPase
cat1	MGG_13279	0	52	K/Na-ATPase
cat2	MGG_05078	0	69	Probably K/Na-ATPase
cat3	MGG_12005	0	75	Unknown pump specificity
cat4	MGG_06295	0	58	Unknown pump specificity
exc1	MGG_01638	0	41	Na^{+}/Ca^{2+}-exchanger
exc2	MGG_11454	0	52	Vacuolar Ca^{+} transporter
exc3	MGG_08710	0	27	Vacuolar Ca^{+} transporter
exc4	MGG_04159	0	54	Vacuolar Ca^{+} transporter
exc5	MGG_01381	0	53	Calcium permease
hyp1	MGG_06475	0	64	Hypothetical protein; Could be related to Ca^{2+} signalling
anxA7	MGG_06847	0	63	Annexin A7
hyp2	MGG_00654	0	48	Hypothetical protein; EF hand domain-containing protein
ndeh1	MGG_04140	0	66	NADH dehydrogenase; Could be related to Ca^{2+} signalling
sal1	MGG_01072	0	53	Ca^{2+}-binding mitochondrial carrier
hyp3	MGG_02342	0	49	Hypothetical protein; Could be related to Ca^{2+} signalling
ara1	MGG_07066	0	71	Ca^{2+}-binding mitochondrial carrier protein
sagA	MGG_06180	0	76	Contains EF hands
cdc4	MGG_09470	0	58	Myosin regulatory light chain cdc4
ndeh2	MGG_06276	0	65	NADH dehydrogenase; Could be related to Ca^{2+} signalling

(continued)

Table 4 (continued)

Gene name	*M. oryzae* locus	E value	% Identity	Comment
dun1	MGG_01596	0	58	DNA damage-response kinase
rck2	MGG_08547	0	69	Ca^{2+}/Calmodulin-dependent protein kinase-like protein
hyp4	MGG_03548	0	30	Putative tropomiosin-1 alpha chain
cnx	MGG_01607	0	72	Calnexin
hyp5	MGG_08154	0	46	Calmodulin like protein
kin4	MGG_01196	0	47	Could be related to calcium signalling

E values for the BLASTP analysis and the amino acid sequence identity between the *M. oryzae* protein and the predicted polypeptide sequence for the *E. festucae* homologue are given. Proteins chosen for this analysis were based on those present in the *M. oryzae* genome (Nguyen et al. 2008). Other calcium signalling genes may be present in the *E. festucae*. *E. festucae* gene models are available on request

the cellular response to stress induced by high salt concentration (Cunningham and Fink 1996; Park et al. 2001). PMR1 homologues in *A. niger* and *Aspergillus fumigatus* are required for maintaining Ca^{2+} homeostasis in these fungi (Yang et al. 2001; Pinchai et al. 2010). However, *A. fumigatus pmrA*, encoding a Ca^{2+} ATPase, is not required for pathogenicity (Pinchai et al. 2010). *E. festucae* contains three PMC1 (vacuolar Ca^{2+} ATPases) and two PMR1 (Golgi Ca^{2+} ATPases) homologues, and five Ca^{2+} exchangers (Table 4).

Phospholipase C

In *S. cerevisiae*, deletion of *PLC1* was shown to have pleiotropic effects, including variable rates of survival depending on the genetic background (Yoko-o et al. 1993). Mutants display sensitivity to osmotic stress, temperature stress and show defects in the utilisation of carbon sources other than glucose (Flick and Thorner 1993). PLC1 works together with the Gpr1p/Gpa2p GPCR to transduce glucose-induced calcium signals (Flick and Thorner 1993; Tisi et al. 2002). In *M. oryzae*, disruption of *plc1* resulted in reduced pathogenicity, presumably due to disturbance of calcium fluxes essential for the infection process (Rho et al. 2009). *E. festucae* contains four PLC homologues (Table 4). Given the pathogenicity defects of PLC1 mutants of *M. oryzae* the homologue from *E. festucae* may be important for signalling and maintenance of the symbiosis.

Calmodulin

The calcium sensor calmodulin is involved in many cell processes through the regulation of targets such as the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin or Ca^{2+}/calmodulin dependent protein kinases. The *S. cerevisiae* calmodulin gene, *CMD1*, is essential for viability (Davis et al. 1986). While little is

known about the role calmodulin plays in phytopathogenicity, expression of *M. oryzae* calmodulin is upregulated during appressorium formation (Liu and Kolattukudy 1999). Additionally, calmodulin preferentially localises to germ tubes and appressoria, suggestive of a role in the infection process (Ma et al. 2009). In support of this, silencing of calmodulin was found to dramatically reduce pathogenicity of *M. oryzae*. *E. festucae* contains one, highly conserved CAM homologue (Table 4).

Ca^{2+}/Calmodulin-Dependent Protein Kinases

An important group of calcium-regulated enzymes is the Ca^{2+}/calmodulin-dependent protein kinases (CaMKs). CaMKs are serine/threonine protein kinases with an amino terminal catalytic domain and a carboxy-terminal regulatory domain. The regulatory domain consists of an autoinhibitory region and the Ca^{2+}/calmodulin (Ca^{2+}/CaM) binding region. The autoinhibitory domain regulates the activity of the enzyme by interacting with the catalytic domain to prevent substrate binding or distort the catalytic site. Binding of Ca^{2+} activated calmodulin with the calmodulin-binding domain results in a conformational change that releases the catalytic domain to overcome this autoinhibition. After the initial activation, the enzyme may remain active independently of the presence of Ca^{2+}/CaM. Furthermore, the enzyme may require additional modifications, such as phosphorylation, for full activation. Fungal CaMKs play important roles in fungal development as well as interactions with other organisms. *S. cerevisiae* contains two CaM kinases, CMK1 and CMK2 (Ohya et al. 1991). These are involved in thermotolerance (Iida et al. 1995) and cell survival following pheromone-induced cell cycle arrest (Moser et al. 1996). In the filamentous fungus *A. nidulans,* three Ca^{2+}/CaM-dependent protein kinases, CmkA, CmkB and CmkC, have been reported. CmkA and CmkB are *bona fide* CaMKs, whereas CmkC is a CaMKK, which phosphorylates CmkB in vitro. Disruption of *cmkA* or *cmkB* is lethal, while expression of a constitutively active form of CmkA prevents spores from entering the first nuclear division cycle (Dayton and Means 1996; Dayton et al. 1997; Joseph and Means 2000). In *N. crassa*, deletion of *CAMK-1* induces a transient slow growth phenotype (Yang et al. 2001). CaM kinases also appear to play a role in phytopathogenicity. *M. oryzae cmk1* mutants have delayed spore germination and appressorium formation, leading to a reduced ability to infect host plants (Liu et al. 2009). Additionally, inhibitors of CaMK block the *Colletotrichum gloeosporioides* infection process (Kim et al. 1998). However, not all CaM kinases are essential for phytopathogenicity, as loss of any of the three CaMKs of *Stagnospora nodorum* does not prevent pathogenicity (Solomon et al. 2006). *E. festucae* contains homologues of *A. nidulans cmkA*, *cmkB* and *cmkC* (Table 4). However, none of these genes appear to be required for establishment or maintenance of the symbiotic interaction between *E. festucae* and perennial ryegrass (Mitic 2011).

Calcineurin

Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase (PP2B) comprised of a catalytic subunit (CnA) and a regulatory subunit (CnB). The catalytic subunit is a polypeptide with four different domains: catalytic domain, CnB binding domain, Ca^{2+}/CaM binding domain and an autoinhibitory domain. As for CaMKs, the autoinhibitory domain inhibits enzyme activity and this inhibition is relieved by Ca^{2+}/CaM binding (Rusnak and Mertz 2000). Calcineurin signalling, which occurs via the calcineurin-responsive transcription factor CRZ1, is important for regulation of ion stress, cell wall integrity, hyphal growth and a number of other developmental processes (Stie and Fox 2008). Calcineurin signalling is also important for many fungal–plant associations. In *U. maydis,* calcineurin is essential for virulence, with mutants unable to form tumours (Egan et al. 2009). In *M. oryzae*, treatment with the calcineurin inhibitor cyclosporin A blocks appressorium formation, and RNAi silencing of the calcineurin A gene, *MCNA*, significantly reduces appressorium formation (Choi et al. 2009b). Disruption of *M. oryzae CRZ1* confirmed that calcineurin signalling is required for the infection process, as mutants seldom developed infectious hyphae (Choi et al. 2009a), possibly due to the reduced turgor pressure of the mutant compared to wild type (Zhang et al. 2009). *B. cinerea crz1* mutants are defective in penetration, but this can be rescued by addition of Mg^{2+} (Schumacher et al. 2008). An analysis of the genome sequences of *E. festucae* strains E2368 and E984 (Fl1) identified two copies of *cnaA* in the former and just one in the latter (Table 4). A *cnaA* deletion mutant of Fl1 induced a severe HR-like response when seedlings of perennial ryegrass were infected with this mutant (Mitic 2011), suggesting calcineurin signalling does play a role in symbiotic maintenance.

5 Host Defence Response

Plants recognise potentially pathogenic microorganisms, such as bacteria and fungi, and react to their presence by activation of a multi-layered host defence mechanism. Activation of the first layer of the plant immune response depends on recognition of pathogen-associated molecular patterns (PAMPs). Perception of PAMPs by the plant activates PAMP-triggered immunity (PTI), which, among other responses, induces rapid ion flux across the membrane, ROS production, changes in gene expression and cell wall reinforcement (Zipfel 2008). Many pathogens have evolved various effector proteins that can interfere with PTI, thereby allowing the infection process to continue. NBS-LRR proteins are key for plant recognition of microbial effector proteins. This recognition is species specific and results in effector-triggered immunity (ETI). ETI usually involves host cell death that is triggered by a hypersensitive response (Jones and Dangl 2006). Chitin is the best studied fungal PAMP. Suppression of a gene encoding a chitin receptor in rice, *CEBiP*, leads to a reduction in the oxidative burst response and expression of genes that normally define a plant chitin-induced response (Kaku et al. 2006).

Symbiotic fungi, such as endophytes and mycorrhizal fungi need to avoid host defences in order to establish a functional symbiosis. However, it remains unclear how this avoidance is achieved in different fungi. Based on a PTI/ETI host response model, avoidance of host defences could be achieved by masking of fungal PAMPs or through suppression of host immune responses triggered by fungal effectors. It is also possible that the plant itself prevents a defence response upon specific recognition of the symbiotic partner. Indeed, transcriptional analysis of the association between *Pinus sylvestris* and the ectomycorrhizal fungus *Laccaria bicolor* revealed an initial upregulation of some defence and cell wall biosynthesis related genes, followed by a downregulation of these genes in the later stages of mycorrhizal establishment (Heller et al. 2008).

In epichloë endophyte associations it remains unclear how the fungus evades any host defence response – whether the fungus is able to somehow "hide" from the host, or whether the host recognises the fungus but does not mount a response. While wild-type *E. festucae* does not induce a host defence response some *E. festucae* symbiotic mutants that form pathogenic associations with perennial ryegrass do activate host defences. High throughput mRNA sequencing of perennial ryegrass associations with *E. festucae*-wild-type and a MAP kinase mutant strain (Δ*sakA*), revealed a dramatic up-regulation of host defence-related genes including pathogenicity response (PR) genes and NBS-LRR genes (Eaton et al. 2010). It is unclear what changes to the Δ*sakA* mutant induced these host defence processes. A possible explanation for the host defence response seen in plants infected with the Δ*sakA* mutant is a change in the fungal cell wall composition or structure that allows detection by the host. Although the composition of the cell walls of wild-type *E. festucae* and the Δ*sakA* mutant have yet to be investigated, transmission electron micrographs show that the cell walls of the Δ*sakA* mutant hyphae in the host pseudostem often appear much thinner, and more diffuse than those of wild type, possibly indicating a change in cell wall composition (Eaton et al. 2010).

6 Signalling Associated with Synthesis of Bioprotective Metabolites

Four main classes of biologically active metabolites have been identified in grass hosts infected with *E. festucae*: peramine, indole diterpenes (principally lolitrem B), ergot alkaloids (principally ergovaline) and lolines. Peramine is a potent feeding deterrent of adult Argentine stem weevil (ASW; *Listronotus bonariensis*), an economically important pest of *L. perenne* in New Zealand agricultural ecosystems. Lolitrem B also has biological activity against some insect larvae but is better known as the causative agent of "ryegrass staggers", a neuromuscular disorder that affects animals grazing on pastures with high toxin levels. Although the fitness benefits of ergot alkaloids are still to be defined, they are implicated in herbivory protection against some insects and nematodes. However, ergot alkaloids are best known for

the livestock grazing disorder, "fescue toxicosis". Loline alkaloids have potent anti-insecticidal properties and can accumulate to very high levels in grass tissues.

Genes for the synthesis of peramine (Tanaka et al. 2005), indole diterpenes (Young et al. 2005, 2006), ergot alkaloids (Panaccione et al. 2001; Wang et al. 2004; Fleetwood et al. 2007) and lolines (Spiering et al. 2005) have been cloned and the biochemical function of the protein products inferred from a combination of chemical and genetic analysis and by a comparison with the known functions of homologues in other filamentous fungi. With the exception of peramine, which appears to require just a single gene product, PerA, for its synthesis, the other classes of metabolites require multiple gene products, encoded by sets of genes that are organised in distinct clusters within the *E. festucae* genome. At least ten genes are required for lolitrem (*ltm*) and related indole diterpene biosynthesis (Young et al. 2006), ten genes are required for ergot alkaloid biosynthesis (*eas*) (Schardl et al. 2006; Fleetwood et al. 2007) and nine genes are required for loline (*lol*) (Spiering et al. 2005) biosynthesis. Using the *E. festucae*–perennial ryegrass symbiotic association as a model experimental system we have shown that the *per*, *eas* and *ltm* genes are all preferentially and highly expressed in planta, but not expressed in culture, even in the presence of crude plant extracts (Tanaka et al. 2005; Young et al. 2005, 2006; Fleetwood et al. 2007; May et al. 2008). When a promoter of the *ltmM* (P*ltmM*) gene was fused to the *gusA* reporter, GusA activity was detected in most vegetative and reproductive tissues, including the developing seed, but not in axenic *E. festucae* cultures (May et al. 2008). Taken together these results provide strong evidence that plant-specific signalling is required for expression of all three biosynthetic pathways. With the exception of *N. uncinatum*, lolines have not been detected in axenic cultures of epichloë endophytes (Blankenship et al. 2001). However, even in *N. uncinatum*-containing symbiota the *lol* genes are more highly expressed in planta compared to culture, confirming that these genes are also symbiotically regulated.

A distinctive structural feature of these *E. festucae* secondary metabolite clusters is their association with a complex array of Type I (RNA based) and Type II (DNA based) transposon relics, sequences known to promote a chromatin state that is transcriptionally silent (Martienssen and Colot 2001). Recent work has shown that chromatin remodelling is required for activation of secondary metabolite biosynthesis in *Aspergillus* spp. (Shwab et al. 2007; Bok et al. 2009; Reyes-Dominguez et al. 2010). Therefore, chromatin remodelling is predicted to be an important component of *E. festucae* secondary metabolite gene activation in planta. How this occurs in response to plant host signals is a key question to understanding the symbiosis.

7 Future Challenges

A key challenge in the future will be identifying plant metabolites and their associated receptors which sense and transduce these signals to activate (1) ROS expression and (2) secondary metabolite biosynthesis, in order to control hyphal

growth and regulate synthesis of bioprotective molecules to maintain a mutually beneficial symbiotic interaction. The crucial cellular zone for interaction between symbiont and host is the apoplast. It is therefore important that we gain a better understanding of the metabolome and proteome of the grass apoplastic space, in the presence and absence of endophyte, to identify candidate metabolites and proteins that are involved in signalling between the two partners. A defining feature of this symbiotic interaction is the tight attachment of hyphae to the host cell wall (Fig. 2). This intimate cellular connection provides an ideal passage for nutrient and signal exchange. The fungal plasma membrane at this interface will contain a suite of transporters and receptors that respond to various plant signals. Strategies that could be used to refine which of these transporters and receptors are crucial for symbiotic maintenance include RNAseq of mutant versus wild-type symbiota (Cox et al. 2010; Eaton et al. 2010), high throughput metabolite screens using *E. festucae* symbiotic gene promoters fused to EGFP (Gardiner et al. 2009), combined with ligand binding assays, and various protein–protein interaction assays. RNAseq also has the potential to identify the suite of transcriptional regulators that are associated with the symbiotic regulatory network. A comparison of the transcriptomes of *sakA* mutant versus wild-type symbiosis identified a large set of genes encoding putative transcription factors that were differentially expressed, including $Zn(II)_2Cys_6$ binuclear cluster, basic region-leucine zipper (bZIP) and Zn(II) coordinating Cys_2His_2 factors (Eaton et al. 2010). Targeted deletion of the genes for these putative transcription factors combined with plant phenotype testing will determine if these genes are required for symbiotic maintenance. Genes that affect secondary metabolite biosynthesis or the symbiotic interaction with the host could be further analysed by a combination of ChIPseq and RNAseq to identify the downstream effectors of these transcriptional regulators. These combined approaches will provide important insights into signalling in the *E. festucae–L. perenne* symbiosis and help understand the differences between fungal mutualistic and pathogenic interactions with plants.

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Plant Infection by Biotrophic Fungal and Oomycete Pathogens

Pamela H.P. Gan, Peter N. Dodds, and Adrienne R. Hardham

Abstract Biotrophic fungi and oomycetes constitute some of the most destructive pathogens of agriculturally important plants. In susceptible hosts, these pathogens infect and establish a dynamic relationship with living plant cells through which they redirect plant resources to support pathogen growth and reproduction. During evolution, biotrophic infection strategies have become finely tuned to allow the pathogen to avoid or suppress the plant's defence response and to take control of host cell organisation and metabolism. Recent research has begun to uncover intriguing details of pathogen effector proteins that are instrumental in facilitating biotrophic growth. These effectors function in both the plant apoplast and cytoplasm. They enable plant penetration, they mask factors that normally trigger basal defence and they suppress the host defence response. Effectors responsible for redirecting host metabolism remain to be characterised. Ongoing research efforts focus on elucidation of effector identity and function and of the mechanisms that operate in the uptake of intracellular effectors into the host cytoplasm.

1 Introduction

Plants constantly come into contact with a diverse range of micro-organisms, be they viruses, bacteria, fungi or oomycetes. Nevertheless, in the majority of cases they are able to fend off infection by pathogens, making disease the exception rather than the rule. In the case of fungi, fewer than 2% of the 100,000 fungal

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species known to exist actually cause disease in living plants (Agrios 1997). Of the rest, some are mutualistic, as described in other chapters of this text, but most are saprophytes that are strictly involved in the decomposition of plant material that is already dead. Furthermore, those micro-organisms that are pathogenic can infect only a limited number of host plant species (their host range). There is an on-going evolutionary battle, with individual plants being susceptible to certain pathogenic micro-organisms and individual pathogens able to infect only certain plant species or cultivars. Thus, while resistance to microbial attack has evolved in most plants, the same evolutionary forces have given rise to pathogens capable of overcoming plant defences, often with devastating results. This chapter presents our current understanding of the strategies fungal and oomycete pathogens use to infect plants and establish disease.

Fungi and oomycetes are two major groups of eukaryotic plant pathogens. They share many morphological similarities that are likely to have arisen through convergent evolution as efficient mechanisms for development and reproduction, including growth as filamentous hyphae and production and dispersal of sexual and asexual spores (Latijnhouwers et al. 2003). Early plant pathologists considered oomycetes to be a class of organisms within the fungal kingdom, however, structural and biochemical differences and comparisons of nucleotide sequences have provided strong evidence that the two groups are phylogenetically separate (Hardham 2005; Van de Peer and De Watcher 1997). It is now recognised that the oomycetes are more closely related to the coloured algae and alveolates, the latter including apicomplexan parasites such as *Plasmodium falciparum*, the causal agent of malaria (Baldauf et al. 2000), than they are to true fungi.

Fungal and oomycete plant pathogens have two main life-styles. Necrotrophic pathogens infect living plants but quickly kill plant tissues, obtaining nutrients from dead or dying cells. Biotrophic pathogens, on the other hand, establish a stable relationship with living plant cells, obtaining nutrients by manipulating host cell metabolism. Some pathogens, such as *Ustilago maydis*, the causal agent of corn smut disease, are facultative biotrophs, being also capable of host-independent growth. Others, such as the rust fungi, powdery mildews and downy mildews, are obligate biotrophs, only growing and reproducing in association with a host plant. In addition, a number of fungal and oomycete plant pathogens have an intermediate life-style known as hemibiotrophy, where the pathogen initially infects and propagates in living host tissue before switching to a necrotrophic phase of disease development. Examples of hemibiotrophs include the rice blast fungus, *Magnaporthe oryzae*, some *Colletotrichum* species and the oomycetes *Phytophthora infestans*, *P. sojae* and *P. nicotianae*.

2 Infection Strategies

In order to infect plants, pathogens must overcome a range of highly effective constitutive and induced, physical and chemical plant defences. In most cases, the infection cycle involves four key stages – adhesion to the plant surface, penetration

and colonisation of the plant, acquisition of nutrients and reproduction. Details of the strategies followed in achieving these goals vary between different groups of pathogens. A brief overview of the infection processes employed by the major groups of biotrophic fungal and oomycete plant pathogens follows, with a particular focus on penetration and colonisation.

2.1 Rust Fungi

Rust fungi belong to the Basidiomycetes and are some of the most successful obligate biotrophs. A number have served as model systems for investigations of plant–pathogen interactions. For example, genetic studies of *Melampsora lini* (flax rust) formed the basis of the gene-for-gene hypothesis which states that every resistance gene in a plant has a corresponding avirulence (Avr) gene in the pathogen (Flor 1955). Molecular studies of *Uromyces fabae* (broad bean rust) have contributed to our understanding of nutrient acquisition during biotrophic growth. Due to their major contribution to disease development and propagation, dikaryotic asexual rust uredospores have been the focus of most studies of rust infection strategies (Fig. 1). When a uredospore lands on a plant, it adheres to the plant surface initially through passive, hydrophobic interactions before secreting material that forms an adhesive pad of glycoproteins and carbohydrates (Clement et al. 1993; Deising et al. 1996). Upon spore germination, the germ tube extends by tip growth across the leaf surface. The detection of chemical signals on the plant surface is no doubt a feature common to most, if not all fungi and oomycetes, but hyphae of rust fungi also sense and respond to topographical signals from the underlying surface. During this thigmotropic growth, hyphae of *Puccinia* species, for example, extend in a direction that is perpendicular to the long axis of the leaf epidermal cells (Read et al. 1992; Staples and Hoch 1997). When the hypha encounters a guard cell, the hyphal apex swells and differentiates into an appressorium over the stomatal pore (Hoch and Staples 1991). Both thigmotropic growth and appressorium formation can be induced on inert replicas of the leaf surface or artificial gratings providing clear evidence that the inductive signals are associated with topographical features of the underlying surface (Hoch et al. 1987). Entry of the fungus into the plant leaf then occurs through the stomatal cavity via a penetration hypha produced from the base of the appressorium.

On entering the leaf tissue, the penetration hypha expands to form a substomatal vesicle in the substomatal cavity. From this vesicle, one or more primary infection hyphae grow until they make contact with a host mesophyll cell whereupon they differentiate to form haustorial mother cells (Heath and Skalamera 1997). A penetration hypha is formed by the haustorial mother cell and grows through the plant cell wall, invaginating the host plasma membrane as it expands and differentiates into a haustorium (Harder and Chong 1991). Upon establishment of haustoria, the fungus is no longer metabolically independent and enters a “parasitic/

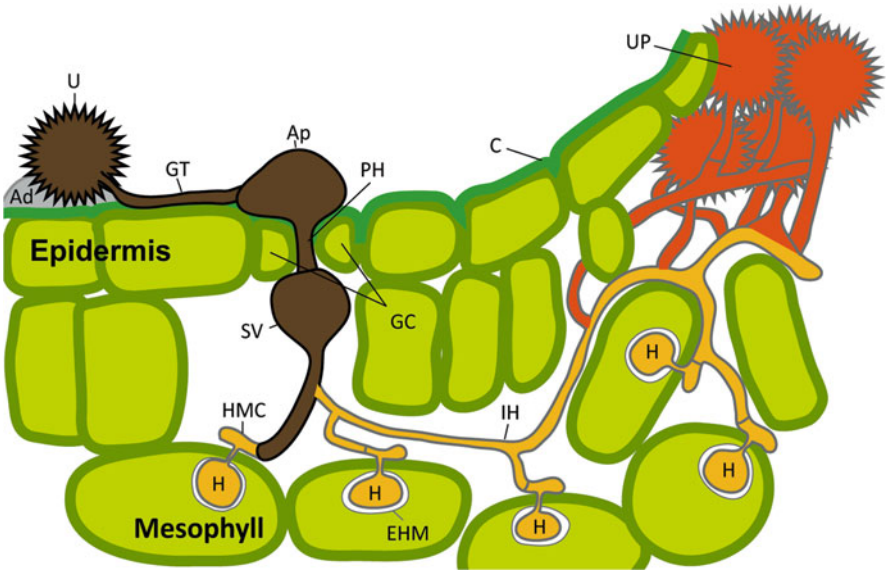

Fig. 1 Schematic representation of the rust fungal uredospore infection. Diagram design modified from Voegele (2006). *U* uredospore, *Ad* adhesive, *GT* germ tube, *Ap* appressorium, *PH* penetration hypha, *SV* substomatal vesicle, *HMC* haustorial mother cell, *H* haustorium, *EHM* extrahaustorial membrane, *GC* stomatal guard cell, *IH* infection hyphae, *C* cuticle, *UP* uredospore pustule. Early infection structures from the "penetration stage" are shown in *dark brown*, structures from the "parasitic/biotrophic stage" are shown in *yellow* and structures from the "sporulation stage" are shown in *red*

biotrophic phase" during which it obtains nutrients from its host (Deising et al. 1996; Voegele 2006).

2.2 Powdery Mildews

Powdery mildews are biotrophic ascomycetes that infect aerial parts of higher plants. After landing on a plant, asexual conidia attach to the plant surface and form one or, in the case of *Blumeria graminis*, two germ tubes (Fig. 2). In *B. graminis*, growth of the first, the primary germ tube, is limited, while the second, the appressorial germ tube, extends across the leaf, often growing along the longitudinal grooves between adjacent epidermal cells (Carver et al. 1995). The primary germ tube is believed to function in attaching the germling to the plant surface, in obtaining water from the host, and in perception of chemical and physical signals from the plant (Green et al. 2002). Signal transduction by the primary germ tube influences subsequent germination and growth of the appressorial germ tube. In response to inductive signals, the appressorial germ tube swells at its tip and differentiates into a lobed appressorium which, via a

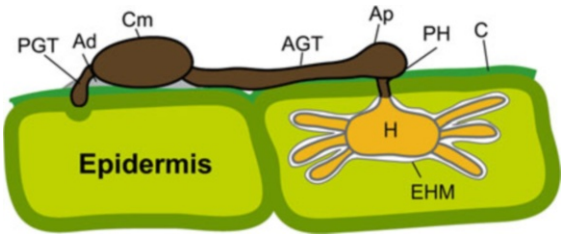

Fig. 2 Schematic representation of *Blumeria graminis* infection. *Ad* adhesive, *Ap* appressorium, *PH* penetration hypha, *H* haustorium, *EHM* extrahaustorial membrane, *PGT* primary germ tube, *Cm* conidium, *AGT* appresorial germ tube, *C* cuticle. Early infection structures from the "penetration stage" are shown in *dark brown* and structures from the "parasitic/biotrophic stage" are shown in *yellow*

penetration hypha, grows through the underlying host cuticle and cell wall (Green et al. 2002). In contrast to rusts, powdery mildew appressoria form on the outer periclinal wall of epidermal cells and directly penetrate the host cell wall. After breaking through the cell wall, the penetration hypha develops into a haustorium possessing finger-like projections that, as in rust infections, invaginate the plasma membrane of the living host cell.

2.3 Magnaporthe oryzae

Magnaporthe oryzae is best known for the devastating blast disease it causes on rice leaves (Talbot 2003) but it is also able to infect the roots of rice plants (Sesma and Osbourn 2004; Marcel et al. 2010; Tucker et al. 2010). Depending on the host, symptoms of *M. oryzae* infection can range from no visible symptoms to larger necrotic lesions of different sizes indicating different proportions of necrotrophic and biotrophic growth in fungal infection (Heath et al. 1991). Tissue-specific differences in feeding strategies also exist. During foliar infections of rice, *M. oryzae* is a hemibiotroph which infects and grows in living plant cells but, after spreading to adjacent cells, it kills the initially infected cells (Wilson and Talbot 2009; Kankanala et al. 2007). In rice roots, *M. oryzae* grows only biotrophically (Wilson and Talbot 2009).

During foliar infection (Fig. 3), after landing on the plant, conidia of *M. oryzae* release spore-tip mucilage which glues the spore to the plant surface (Hamer et al. 1988). The conidium germinates and the tip of the emergent germ tube swells and differentiates into a dome-shaped appressorium. Cytoplasm flows from the conidium into the appressorium as it enlarges and by the time the latter is mature, the conidium is empty and collapsed (Braun and Howard 1994). The flat base of the appressorium attaches tenaciously to the plant epidermis. The domed walls of the cell become melanised, thereby greatly reducing permeability of the appressorial wall and allowing passage of only water molecules (Howard et al. 1991).

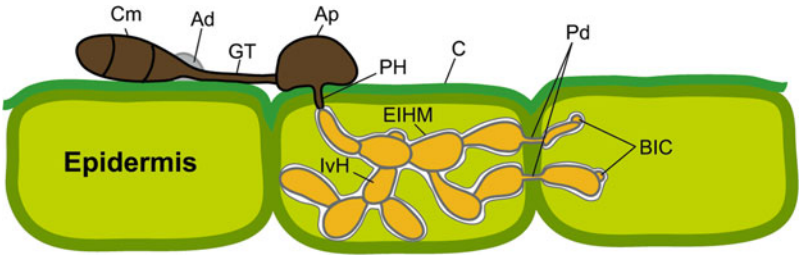

Fig. 3 Schematic representation of *Magnaporthe oryzae* foliar infection. *Ad* adhesive, *GT* germ tube, *Ap* appressorium, *PH* penetration hypha, *Cm* conidium, *IvH* invasive hyphae, *EIHM* extra-invasive hyphal membrane, *Pd* plasmodesmata, *BIC* biotrophic interfacial complex, *C* cuticle. Early infection structures from the "penetration stage" are shown in *dark brown* and structures from the "parasitic/biotrophic stage" are shown in *yellow*

Concomitant accumulation of high concentrations of glycerol generates turgor pressures as high as 8 MPa within the cell (Howard et al. 1991). A penetration hypha containing actin microfilaments forms at the base of the appressorium and breaches the underlying plant cell wall using both physical force and localised enzymatic degradation of wall components (Howard et al. 1991). Within the epidermal cell, *M. oryzae* forms bulbous invasive hyphae that are surrounded by a host-derived extra-invasive hyphal membrane (Kankanala et al. 2007). Invasive hyphae form a specialised structure termed the biotrophic interfacial complex which is involved in secretion of some virulence factors (Khang et al. 2010). Invasive hyphae grow into neighbouring cells via plasmodesmata in the intervening cell wall and at this time, the previously infected cell dies (Kankanala et al. 2007).

During root infection, *M. oryzae* conidia germinate from both terminal cells forming preinvasive hyphae and hyphopodia on the root surface (Tucker et al. 2010). Hyphopodia are hyphal swellings that, like appressoria, may aid host cell penetration. Invasive hyphae are produced by both surface hyphae and hyphopodia and breach the outer epidermal cell wall. The infection remains biotrophic with no evidence of a switch to a necrotrophic phase (Marcel et al. 2010).

2.4 Colletotrichum *Species*

Most species of *Colletotrichum* are hemibiotrophs that grow biotrophically in living plant cells for 1 to a few days before adopting a necrotrophic life-style. Like the powdery mildews and *M. oryzae*, most *Colletotrichum* species penetrate the host cuticle and cell wall of epidermal cells directly, rather than entering through a stomatal aperture (Fig. 4; Willmer and Fricker 1996; Mendgen and Deising 1993).

Colletotrichum conidia germinate on the surface of a host and form a short germ tube which responds to inductive signals on the plant surface and differentiates into a dome-shaped appressorium. Structural and biochemical specialisation of

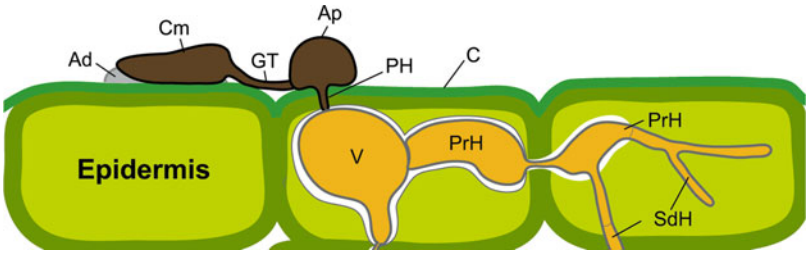

Fig. 4 Schematic representation of *Colletotrichum lindemuthum* infection on a compatible host. In other *Colletotrichum* species (e.g. *Colletotrichum destructivum*), only secondary, necrotrophic hyphae spread from the initial invaded cell. Image design modified from Mendgen and Hahn (2002). *Ad* adhesive, *GT* germ tube, *Ap* appressorium, *PH* penetration hypha, *Cm* conidium, *V* vesicle, *PrH* primary hyphae, *SdH* secondary hyphae, *C* cuticle. Early infection structures from the "penetration stage" are shown in *dark brown* and structures from the "parasitic/biotrophic stage" are shown in *yellow*

Colletotrichum appressoria are similar to those of *M. oryzae* – the appressorial cell wall becomes impregnated with melanin and the cell accumulates high concentrations of osmotically active compounds (Mendgen and Deising 1993). In *C. graminicola*, the resultant appressorial turgor pressure has been calculated to exceed 5.2 MPa (Bechinger et al. 1999). As in *M. oryzae*, the appressorial penetration hypha breaches the underlying epidermal cell wall, probably by using a combination of mechanical force and enzymatic cell wall degradation (Münch et al. 2008). Within the epidermal cells, the tip of the penetration hypha swells to form a vesicle and broad primary intracellular hyphae that remain separated from the host cytoplasm by an interstitial matrix and an invaginated host-derived plasma membrane. This relationship lasts for 1–2 days after which the plant plasma membrane is degraded and the infected cell dies. In *Colletotrichum lindemuthianum*, this transient biotrophy is maintained in newly invaded cells but 2–3 days after the onset of infection, narrow secondary hyphae form and breach the plant plasma membrane, killing the host cells. The pathogen thus enters its necrotrophic phase during which secretion of a diversity of cell wall degrading enzymes leads to widespread cell wall digestion (Münch et al. 2008). In *Colletotrichum destructivum*, only secondary necrotrophic hyphae spread from the initial invaded cell (Perfect and Green 2001). In *Colletotrichum capsici*, infection hyphae initially grow between the cuticle and cell wall of the epidermal cells before killing and colonising the plant tissues (Mendgen and Hahn 2002).

2.5 Oomycetes

Oomycete plant pathogens include obligate biotrophs (e.g. the downy mildews), hemibiotrophs (e.g. *P. infestans*) and necrotrophs (e.g. *Phytophthora cinnamomi*). Some are foliar pathogens; others infect plant roots. Hyphae of oomycete species

may penetrate the plant surface via stomata, along the anticlinal wall between adjacent epidermal cells or directly through the outer periclinal cell wall of the epidermis. Biotrophic and hemibiotrophic species form simple, spherical or pyriform haustoria (Enkerli et al. 1997). In hemibiotrophic *Phytophthora* species, tissue colonisation is typically through intercellular hyphal growth with haustoria differentiating within plant cells contacted by the intercellular hyphae (Enkerli et al. 1997).

3 Structure and Function of Fungal and Oomycete Haustoria

Haustoria formed by biotrophic or hemibiotrophic rusts, powdery mildews and oomycetes are central to the development of a stable biotrophic relationship between pathogen and plant and are key sites for the absorption of the plant nutrients that are essential for pathogen growth, development and reproduction. For their function, haustoria depend on highly regulated molecular exchange across the haustorium–plant boundary. Before nutrients begin to move from plant to pathogen across this interface, the pathogen must send a likely plethora of signals into the infected plant cell, molecules that not only orchestrate host cell structure and metabolism but also suppress the plant cell's defence response. Given the importance of communication across this haustorium–plant interface, it is not surprising that the area of this boundary is often increased by the development, in the rusts, of up to five lobes (Littlefield 1972) or, in the powdery mildews, of multiple finger-like projections (Gil and Gay 1977) extending from the main body of the haustorium. There is still much to learn about the structure and function of the haustorium–plant interface but integrated cellular and molecular studies are beginning to reveal details of its molecular specialisations.

The composition of both the haustorial cell wall and plasma membrane may differ from that of the cell wall and plasma membrane of hyphae. In flax rust, for example, monoclonal antibodies identify a component of the haustorial wall not found in hyphal walls of this species (Murdoch and Hardham 1998). The same monoclonal antibodies do not react with haustoria from maize rust (*Puccinia sorghi*) or wheat leaf rust (*Puccinia recondita*), indicating that the haustorial-specific component may also be species-specific (Murdoch and Hardham 1998). Differentiation of haustorial membranes has been demonstrated in the bean rust fungus (see below) and in the hemibiotrophic oomycete, *P. infestans* (Avrova et al. 2008). In *P. infestans*, haustorial membranes, but not membranes of intercellular hyphae, contain the membrane protein, Pihmp1, a protein required for haustoria formation (Avrova et al. 2008).

Haustoria are surrounded by an amorphous, gel-like compartment known as the extrahaustorial matrix, across which molecular exchange between pathogen and plant must occur as shown in Fig. 5 (O'Connell 1987; Hahn and Mendgen 1992; Mendgen and Hahn 2002). The matrix consists of a mixture of components, primarily carbohydrates and glycoproteins, derived from both the fungus and the

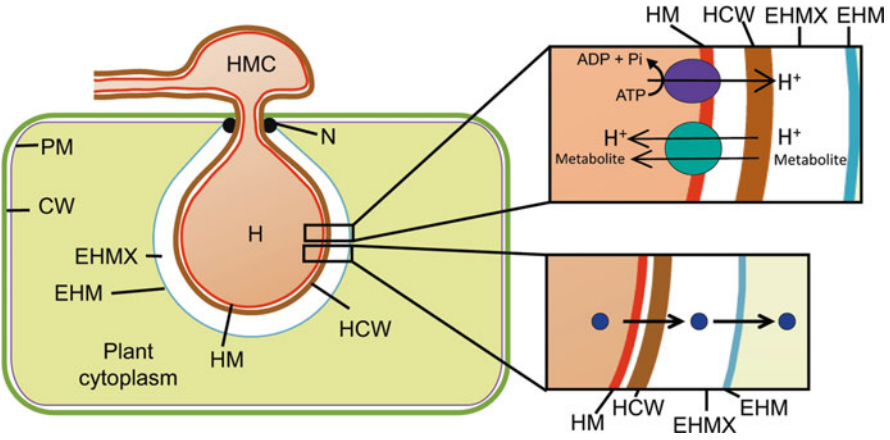

Fig. 5 The plant-haustorium interface. In haustoria-forming biotrophic fungi, the haustorial mother cell (HMC) forms a haustorium (H) surrounded by the extrahaustorial matrix (EHMX), which is a discrete compartment sealed by a neckband (N). Upper inset: A proton gradient generated by ATPases (purple oval) on the haustorial membrane (HM) may facilitate uptake of metabolites into the fungus through proton-driven symporters (*green oval*). Lower inset: Effectors (*blue dots*) may cross the extrahaustorial membrane (EHM), a modified host plasma membrane that invaginates around the haustorium, into the host cytoplasm, where they may interact with target proteins, thus leading to host manipulation or resulting in recognition by cognate resistance proteins and the mounting of a defence response. Effectors may also be secreted into the apoplast. *PM* plant plasma membrane, *CW* plant cell wall, *HCW* haustorial cell wall

plant (Harder and Chong 1991). The extrahaustorial matrix is delimited on the pathogen side by the haustorial cell wall and on the host side by an extension of the host plasma membrane known as the extrahaustorial membrane (Harder and Chong 1991). In rust and powdery mildew infections, the extrahaustorial matrix is sealed off from the apoplast by a structure known as the neckband which is thought to act as a selectively permeable barrier, like the Casparian strip at the endodermis of plant roots (Heath 1976; Harder and Chong 1991). Haustoria of hemibiotrophic fungi and oomycetes appear to be less differentiated than those of the rusts and powdery mildews and generally lack neckbands, although some exceptions exist, as in the case of the oomycete *Albugo candida* which possesses a simple neckband (Soylu 2004; Perfect and Green 2001). In infections by some rust fungi, such as *Puccinia hemerocallidis* and *Puccinia striiformis*, tubular beaded elements contiguous with the extrahaustorial matrix extend from the extrahaustorial membrane into the host cytoplasm, perhaps again serving to increase the surface area of this interface (Mendgen et al. 1991; Mims et al. 2002, 2003). These structures were not observed at the host–haustorial interface during *P. recondita* or *Uromyces appendiculatus* rust infection (Mendgen et al. 1991). Tubular extensions into the cytoplasm from the extrahaustorial membrane also occur during infections by the oomycete *A. candida* (Baka 2008).

Although contiguous with the plant plasma membrane, from which it is thought to be derived, the extrahaustorial membrane exhibits distinct molecular properties

(Hardham 2007). Differences include a reduced glycolipid content, differential staining by phosphotungstic acid and a reduced level of ATPase activity compared to the rest of the host plasma membrane (Baka et al. 1995; Perfect and Green 2001). The extrahaustorial membrane formed during infection of pea plants by the pea powdery mildew, *Erysiphe pisi*, contains a large (200 kDa) glycoprotein that does not occur elsewhere in the plasma membrane of infected cells (Micali et al. 2008; Roberts et al. 1993). Similarly, the plant resistance protein RPW8.2 is specifically targeted to the extrahaustorial membrane of Arabidopsis leaf cells infected by powdery mildew, *Golovinomyces orontii* (Micali et al. 2010). During infections of Arabidopsis by *G. orontii* and another powdery mildew, *Erysiphe cichoracearum*, host plasma membrane proteins are specifically excluded from the extrahaustorial membrane (Koh et al. 2005; Micali et al. 2010).

Haustoria have been difficult to analyse at a molecular level because they are only formed *in planta*. However, methods to isolate haustoria either by differential centrifugation or affinity purification have been developed (Gil and Gay 1977; Tiburzy et al. 1992; Hahn and Mendgen 1992; Micali et al. 2010) and in recent years have facilitated studies of haustorial proteins and transcriptomes (Hahn et al. 1997; Murdoch and Hardham 1998; Catanzariti et al. 2006; Micali et al. 2010). Early studies led to the proposal that haustoria were specialised feeding structures that played a key role in nutrient acquisition, but definitive evidence was difficult to obtain. Strong evidence to support this hypothesis has come from transcriptome analyses of genes that are preferentially expressed in haustoria of the bean rust fungus, *U. fabae* (Hahn and Mendgen 1997). This study identified two cDNA sequences, AAT1 and AAT2, which have homology to genes encoding amino acid transporters (Hahn and Mendgen 1997). AAT1 is a broad-specificity amino acid transporter (Struck et al. 2002). AAT2 was shown by immunolocalisation to occur specifically in the haustorial membrane, although no amino acid transport activity has yet been described for this protein (Mendgen et al. 2000). Further evidence for a role of *U. fabae* haustoria in nutrient acquisition comes from studies of a haustorially expressed sugar uptake transporter, HXT1p, which transports monosaccharides through a proton symport process when expressed in *Xenopus* oocytes (Voegele et al. 2001; Voegele and Mendgen 2003). Given that ATPases, which are required for the pumping of protons across membranes, are reduced on the extrahaustorial membrane but enriched on the fungal haustorial membrane (Baka et al. 1995; Struck et al. 1996, 1998), this finding suggests that during infection, a proton gradient is generated between the extrahaustorial matrix and the haustorium that is used to drive active transport of nutrients towards the pathogen (Fig. 5 inset; Szabo and Bushnell 2001; Baka et al. 1995; Aist and Bushnell 1991).

In addition to this role in nutrient acquisition, it has also emerged that the secretion of pathogen proteins, known as effectors (described in greater detail below), from haustoria is important in establishing biotrophy by allowing manipulation of host metabolism and defence responses. In agreement with this scenario, compatibility between the host and the pathogen appears to be determined at the onset of haustorial development since spore germination, germ tube growth,

appressorium formation and growth of primary infection hyphae develop on a similar time scale in both susceptible and resistant host plants (Dickinson and Lucas 1982).

4 Intracellular Infection Hyphae

Some biotrophic or hemibiotrophic fungal pathogens, such as *M. oryzae*, *U. maydis* and *Colletotrichum* species, do not produce haustoria but instead form specialised intracellular hyphae that may serve the same or a similar function (Mendgen and Hahn 2002; O'Connell and Panstruga 2006). Like haustoria, these intracellular infection hyphae remain separated from the host cytoplasm during biotrophic growth. Intracellular hyphae of *M. oryzae*, for example, are surrounded by a host-derived plasma membrane, termed the extra-invasive hyphal membrane, and an extra-invasive hyphal matrix (Kankanala et al. 2007). Although no neckband structure has been detected which might seal the space between the fungus and the host membrane, the extra-invasive hyphal matrix appears to be distinct from the plant apoplast since addition of the lipid-binding dye FM4-64 to the apoplast leads to the incorporation of the dye into the plasma membrane of plant cells but not of the intracellular hyphae (Kankanala et al. 2007). In hemibiotrophic pathogens, the switch to necrotrophy is associated with distintegration of the host plasma membrane around the intracellular fungal structures, suggesting that the zone of separation between host and pathogen is important in maintaining a biotrophic relationship (Voegele et al. 2009).

5 Host–Pathogen Specificity

As described in the previous sections, biotrophic and hemibiotrophic fungi and oomycetes are able to grow within living host cells and tissues. In order to maintain this intimate association, the pathogen alters and manipulates the host metabolism extensively. Among the changes that have been documented in host plants during infection are movement of the host nucleus and endomembrane system to surround the extrahaustorial membrane; a redistribution of carbohydrates into infected leaves; and an increase in chlorophyll and photosynthetic activity, leading to the "green island effect" (Scholes and Farrar 1985; Coffey 1972; Leckie et al. 1995; Voegele et al. 2009). These events occur during a compatible interaction but in an incompatible interaction plant cells are able to sense pathogen invasion and react rapidly by mounting a multi-faceted defence response.

Plants are able to detect both chemical and physical cues associated with potential pathogens. Currently, plants are seen to have two major categories of defence response. The first, often termed basal resistance, occurs at the onset of infection and is triggered by recognition of conserved pathogen-derived molecules, such as chitin fragments from fungal cell walls or proteins or glucans from

oomycete cell walls (Cosio et al. 1996; Brunner et al. 2002; Kishimoto et al. 2010). These molecules are collectively referred to as elicitors or pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) and are recognised by plant pattern recognition receptors (PRRs) (Boller and Felix 2009). A number of PRRs have been identified. They include the rice CEBiP receptor that recognises fungal chitin (Kaku et al. 2006) and a 75 kDa protein that binds *Phytophthora* cell wall β-glucans (Mithofer et al. 2000). Detection of PAMPs by PRRs results in a localised basal defence response known as PAMP-triggered immunity (PTI) (Jones and Dangl 2006).

One of the earliest responses observed during PTI is the aggregation of cytoplasm in the host cell adjacent to the pathogen appressorium or invading hypha (Freytag et al. 1994). This is accompanied by reorganisation of the actin cytoskeleton and endomembrane system to focus on the penetration site (Takemoto et al. 2003), and reinforcement of the cell wall by cell wall appositions (papillae) (Aist 1976). Wall apposition formation involves localised deposition of structural wall components and anti-microbial compounds and is achieved by the actin-based targeting of secretion. During infection of barley by the powdery mildew *B. graminis*, for example, multivesicular bodies and vesicles containing hydrogen peroxide and phenolics, accumulate in the host cell at the site of penetration (An et al. 2006). PTI-associated defence responses also include the generation of reactive oxygen species (ROS) and the induction of defence-related genes which may inhibit further growth of the invader (Torres 2010; Schwessinger and Zipfel 2008). As demonstrated during the interaction of *Uromyces vignae* with the non-host broad bean plant, clathrin-mediated endocytosis in the plant is active at this early stage of infection and may function to recycle activated defence-related receptors from the plant surface (Xu and Mendgen 1994; Leborgne-Castel et al. 2010). Similar reorganisation of the actin and endomembrane systems can be triggered by mechanical stimuli but the extent of the defence response evoked has not yet been determined (Gus-Mayer et al. 1998; Hardham et al. 2008).

Virulent (adapted) pathogens are able to avoid or suppress PTI. Avoidance mechanisms include the masking or removal of potential PAMPs. Pre-infection by an adapted pathogen has been shown to permit subsequent growth by non-host pathogens, indicating that a general suppression of basal resistance has occurred (Fernandez and Heath 1991). Adapted pathogens can suppress PTI through the synthesis and secretion of specific effector proteins (Jones and Dangl 2006), as discussed in detail below.

Plants have, in turn, evolved resistance proteins that have the ability to recognise pathogen effectors which, in an incompatible interaction, are often called Avr proteins. Plant resistance proteins have a nucleotide binding (NB) domain and a C-terminal leucine-rich repeat (LRR) region (Dodds and Rathjen 2010). In addition, many resistance proteins also have an N-terminal Toll, interleukin-1 receptor domain or a coiled-coil domain (Dodds and Rathjen 2010). These proteins have structural similarity to proteins that have been found to be important for immunity in animal systems, namely, the Toll receptors in Drosophila and Toll-like receptors in mammals (Staskawicz et al. 2001). Collectively NB-LRR proteins confer

resistance against various pathogens including fungi, oomycetes, bacteria, viruses and insects (Dodds and Rathjen 2010). Recognition of an Avr protein by a corresponding resistance protein results in effector-triggered immunity (ETI), which typically culminates in a form of programmed cell death known as the hypersensitive response (HR) (Dodds and Rathjen 2010; Jones and Dangl 2006). In a limited number of cases, both the plant resistance protein and the corresponding pathogen Avr protein have been identified. For example, genes encoding the M and L6 resistance proteins of flax plants and their corresponding Avr proteins, AvrM and AvrL567, of flax rust, have been cloned and the encoded resistance proteins and Avr proteins shown to interact directly (Catanzariti et al. 2010; Lawrence et al. 1995; Anderson et al. 1997; Dodds et al. 2004, 2006; Catanzariti et al. 2006).

6 Effectors

The discovery of pathogen effectors that can "manipulate host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defence responses (avirulence factors or elicitors)" (Kamoun 2006) has led to intense interest and effector-focused research. Although initially identified slowly and laboriously by positional cloning (Orbach et al. 2000; Dodds et al. 2004; Ridout et al. 2006; Gout et al. 2006), advances in DNA sequencing and bioinformatic technologies now facilitate the prediction of suites of candidate effectors in entire phytopathogen genomes (Torto et al. 2003; Choi et al. 2010). Typically, criteria used to interrogate pathogen genomes include identifying sequences encoding small proteins which are under diversifying selection and which have an N-terminal signal peptide (Mueller et al. 2008; Dean et al. 2005; Kamper et al. 2006; Haas et al. 2009). These search strategies have also been applied to cDNA libraries and EST (Expressed Sequence Tags) databases enriched with sequences from the biotrophic phase of pathogenic biotrophs or from the host–pathogen interface. This approach has, for example, resulted in the identification of effectors AvrM, AvrP4 and AvrP123 from *M. lini* (Catanzariti et al. 2006).

Many important insights into effector biology have been acquired by studying the sequences of predicted effectors. Analysis of the sequences of known *Phytophthora* effectors has, for example, led to identification of a conserved N-terminal RXLR dEER motif in these proteins (RXLR-$X_{5\text{-}21}$-dEER; where upper case letters represent consensus amino acids in 10 out of 11 sequences and lowercase letters represent consensus amino acids in more than half of the genes) (Rehmany et al. 2005; Birch et al. 2006). Using this motif as a search criterion, 150–600 candidate effectors have been identified in the genomes of *Phytophthora sojae* and *Phytophthora ramorum*, the exact number obtained depending on the stringencies of the search settings, such as whether or not the dEER motif is also included and if hidden Markov Model statistics are applied (Win et al. 2007; Tyler et al. 2006). Other motifs that have been identified by sequence comparisons of potential *Phytophthora* effectors include the W-, L- and Y-motifs in the C-terminal

portions of some RXLR effectors (Jiang et al. 2008). The W-motif is required for the HR mediated by the interaction between the Rpi-blb1 resistance protein and ipiO effectors (Champouret et al. 2009) and both W- and Y-motifs are required for suppression of programmed cell death by Avr1b (Dou et al. 2008a).

Another family of *Phytophthora* effectors known as Crinklers was identified by analysis of ESTs encoding secreted sequences (Torto et al. 2003). Members of this gene family in *P. infestans*, *P. sojae* and *P. ramorum* cause necrosis in host plants and contain a conserved LXLFLAK motif at the N-terminus (Haas et al. 2009; Win et al. 2007). Similar analyses of EST sequences isolated from barley epidermal cells containing *B. graminis* powdery mildew haustoria identified a Y/F/WxC-motif in small secreted proteins encoded by this fungus (Godfrey et al. 2010). However, not all pathogen effectors have such readily recognisable motifs and, to date, no highly conserved motif has been associated with other fungal effector sequences [e.g. in flax rust (Rafiqi et al. 2010) and in poplar rust (Joly et al. 2010)]. In the majority of cases, the function and biological significance of the putative effectors identified by bioinformatics remain to be determined. To this end, several high-throughput techniques for functional screening of potential effectors have been developed, including screening for phenotypes arising from expression of proteins in host plants transformed by Agrobacterium (Torto et al. 2003; Oh et al. 2009).

7 Importance of Effectors During Infection

The large number of effectors predicted from sequence analyses hints at the complex nature of pathogen–host interactions. While it is possible that not all of the predicted effectors actually function as such, cohorts of effectors may be required at different stages of infection. For example, the deletion of a potential virulence cluster from the genome of *U. maydis* leads to defects in tumour and spore formation, but does not affect penetration or hyphal growth of the fungus within plant tissue (Kamper et al. 2006; Döehlemann 2011). Different subsets of effectors may also be required according to the age and type of host tissue being infected (Skibbe et al. 2010). Multiple variants of a given effector exist [(e.g. flax rust has six AvrM sequence variants (Catanzariti et al. 2006)], due perhaps to evolutionary selective pressure to evade recognition by host resistance proteins and to retain virulence. Multiple sequence variants may also inhibit effector recognition. In the case of the multigene *P. infestans* ipiO family, normal recognition of the IPI-O1 variant by the RB-resistance protein is impaired in the presence of the IPI-O4 variant, which is not recognised by the RB-resistance protein (Halterman et al. 2010).

Redundant or non-essential effector function may limit the value of knockout or silencing studies, as illustrated by studies in which the flax rust AvrL567 gene was silenced with no apparent effect on pathogen virulence (Lawrence et al. 2010). However, a number of effectors have been shown to interfere with plant basal defence responses. In *C. fulvum*, two effectors inhibit chitin recognition and ensuing induction of PTI. The Avr4 effector protein binds to chitin in the fungal cell wall possibly protecting it from host chitinases (van den Burg et al. 2006), thus

maintaining fungal cell wall integrity and inhibiting release of the chitin-derived PAMPs that trigger PTI. A second *C. fulvum* effector, Ecp6, binds to chitin fragments and effectively competes with host PAMP recognition receptors for chitin binding, again preventing PTI induction (de Jonge et al. 2010). Some effectors appear to inhibit downstream PTI signalling processes. The *P. infestans* Avr3a effector, for example, suppresses cell death induced by the elicitor INF1 in plants that lack the R3a resistance protein (Bos et al. 2006, 2009). Further, during *Fusarium oxysporum* infection of tomato, the effector Avr1 inhibits HR induced by Avr2 and Avr3 (Houterman et al. 2008).

A number of effectors are predicted to target host enzymes that may be involved in plant defence. These include the EPI1 Kazal-like protease inhibitor from *P. infestans* which inhibits the P69B protease, the EPIC2B effector which inhibits host papain proteases PIP1 and RCR3, the *C. fulvum* Avr2 effector which also inhibits RCR3, and the *P. infestans* glucanase inhibitors GIP1 and GIP2 (Tian et al. 2005, 2007; Song et al. 2009; Rooney et al. 2005; van Esse et al. 2008; Shabab et al. 2008; Rose et al. 2002; Damasceno et al. 2008; Hein et al. 2009). The flax rust effector, AvrP123, has also been shown to have homology to Kazal-like protease inhibitors although the biological significance of this has not yet been demonstrated (Catanzariti et al. 2006). According to Kamoun's (2006) definition, the great diversity of cell wall degrading enzymes produced by pathogens constitute extracellular effectors (Hardham and Cahill 2010).

In many cases, the functions of putative effectors are yet to be determined, although many identified effectors are Avr proteins and are thus known to trigger the resistance response when detected by a corresponding resistance protein. Interestingly, mutational analysis of Avr3a indicates that the Avr and virulence functions of the effector can be separated, since mutants that are recognised by the R3a resistance protein but which do not suppress INF1-induced cell death have been isolated (Bos et al. 2009). In the flax-flax rust system, yeast two-hybrid assays show that avirulent but not virulent forms of the AvrL567 and AvrM effectors interact directly with the L6 and M resistance proteins, respectively (Dodds et al. 2006; Catanzariti et al. 2010). AvrPita from *M. oryzae* was also shown to bind the cytoplasmic Pi-ta resistance protein from rice (Jia et al. 2000).

8 Delivery of Effectors into Host Cells

While many of the fungal and oomycete effectors discussed in the previous section are likely to function in the plant apoplast, the direct interaction between Avr proteins and their cognate resistance proteins known to occur within the plant cytoplasm suggests that pathogen effectors must also function in the host cytoplasm.

Early evidence for delivery of fungal proteins into host plant cells came from immunolocalisation of UfRTP1, a haustorially expressed glycoprotein of unknown function secreted by the broad bean rust (Kemen et al. 2005). UfRTP1 was shown to

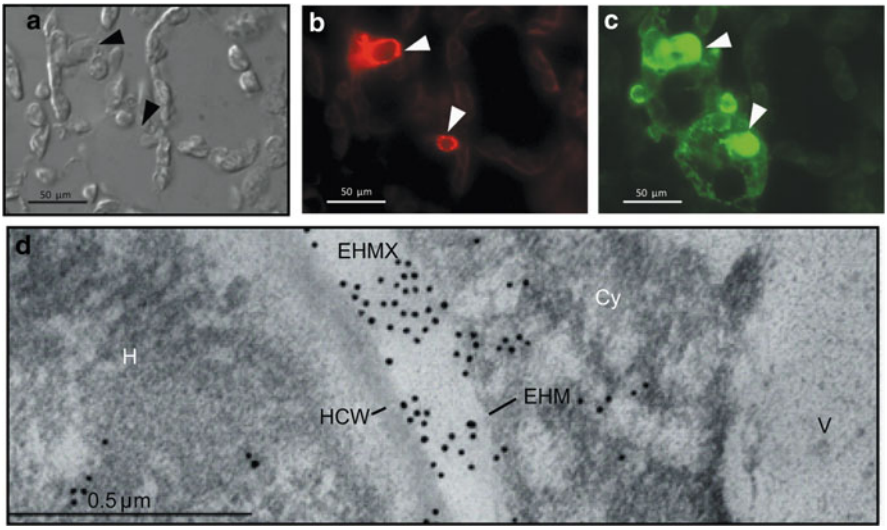

Fig. 6 Localisation of the flax rust effector AvrM *in planta*. (**a**–**c**) Immunofluorescence localisation of AvrM in leaves infected with the flax rust fungus. (**a**) DIC image of fluorescence micrographs showing labelling of haustoria (**b**) and AvrM (**c**). The AvrM signal is strong at and around haustoria (*arrowheads*) but is also detected in the cytoplasm of invaded plant cells. (**d**) Immunogold labelling of AvrM in flax rust-infected leaves. The majority of AvrM labelling is detected in the extrahaustorial matrix (EHMX). However, lower intensity signal was also detected within the plant cytoplasm (Cy). *HCW* haustorial cell wall, *EHM* extrahaustorial membrane

occur in the extrahaustorial matrix and within the host cell during infection (Kemen et al. 2005). UfRTP1 contains a nuclear localisation signal and accumulates in the host nucleus during infection (Kemen et al. 2005). More recently, immunolocalisation experiments with antibodies raised against AvrM have provided direct evidence that this effector is secreted by fungal hyphae and haustoria during infection and enters the cytoplasm of host cells containing one or more haustoria (Fig. **6**; Rafiqi et al. 2010). Localisation of AvrM within the plant cytoplasm is consistent with the recent demonstration of the M resistance protein within the host cytosol (D. Takemoto and D. A. Jones, unpublished observations). Agrobacterium-mediated transient expression of flax rust effectors AvrL567 and AvrM lacking their signal peptides also triggers HR in plants that contain the corresponding resistance proteins, providing further evidence of the function of these proteins within the plant cytoplasm (Dodds et al. 2004; Catanzariti et al. 2006). Expression of wild-type (secreted) AvrL567 and AvrM fused to fluorescent proteins via Agrobacterium-mediated transformation demonstrates that the predicted signal peptides of the fungal effectors are functional in directing protein secretion and that after their secretion from the plant cell, the effectors can re-enter the cytoplasm of both tobacco and flax plant cells (Rafiqi et al. 2010). These experiments provide evidence that uptake of fungal effectors into host plant cells is pathogen-independent, requiring host factors but not pathogen components (Rafiqi et al. 2010). The conservation of

the uptake mechanism between different plant species is consistent with the findings of a study in which purified AvrL567-GFP protein was internalised into soybean root cells and into human A549 cells (Kale et al. 2010).

Localisation of truncation and deletion mutants of AvrM and AvrL567 effector proteins have identified N-terminal regions of less than 31 amino acids that are sufficient to mediate re-uptake of fluorescently tagged protein into host cells (Rafiqi et al. 2010). The primary amino acid sequences of these putative uptake domains are not conserved between the two effectors (Rafiqi et al. 2010). Transient expression of cytoplasmic effectors AvrP123 and AvrP4 from *M. lini* and Avra10 and Avrk1 from *B. graminis* in plants with the corresponding R genes also elicits HR, indicating that delivery of effectors to the plant cytoplasm where they are recognised as part of ETI is a common phenomenon in biotrophic fungal pathogens (Catanzariti et al. 2006; Ridout et al. 2006; Barrett et al. 2009).

Many oomycete effectors also enter the host cytoplasm during infection, but in contrast to the situation with fungal pathogens, studies of the oomycetes have identified two domains involved in transport into the plant cell cytoplasm. In haustoria-forming oomycetes, the N-terminal RXLR motif mediates effector uptake into plant cells (Whisson et al. 2007; Dou et al. 2008b). In oomycete effectors in the Crinkler family, the conserved N-terminal LXLFLAK motif downstream of the signal peptide is required for targeting of Crinkler proteins into host cells (Schornack et al. 2010; Grenville-Briggs et al. 2010). Crinkler effectors are conserved more widely among oomycetes than the RXLR effectors and some necrotrophic oomycetes, such as the legume rot pathogen *Aphanomyces euteiches*, that do not form haustoria possess Crinkler but not RXLR effectors (Schornack et al. 2010). A recent proteomics study has also identified a cell wall-associated Crinkler protein, suggesting that these effectors may function in either the plant apoplast or cytoplasm (Grenville-Briggs et al. 2010). This effector contains the LXLFLAK motif and it remains possible that it might function both intracellularly as well as extracellularly.

As with the flax rust effectors, the mechanism of RXLR effector uptake into host cells is pathogen-independent (Dou et al. 2008b). Purified Avr1b-GFP from *P. sojae* is taken up by soybean root and human A549 cells (Dou et al. 2008b; Kale et al. 2010) and purified SpHTP1-GFP from *Saprolegnia parasitica* is taken up by host fish cells (van West et al. 2010). These results indicate that the mechanism of RXLR entry is not only pathogen-independent but exploits an uptake pathway that is widely conserved among eukaryotic cells.

In *M. oryzae*, which lacks haustoria but forms intracellular invasive hyphae, early studies showed that AvrPita, the effector that interacts directly with the rice cytoplasmic Pi-ta resistance protein, triggered a HR when expressed in the cytoplasm of plants expressing Pi-ta via particle bombardment, suggesting that AvrPita enters host cells where it is recognised by the resistance protein (Jia et al. 2000). Recent studies tracking the delivery of *M. oryzae* effectors into host cells have revealed that a number of pathogen effectors, including AvrPita, PWL1, PWL2 and BAS1 are secreted from the biotrophic interfacial complex into the plant cell cytoplasm (Figs. 1–3; Khang et al. 2010). Initial secretion into the biotrophic

interfacial complex is directed by the signal peptide and/or part of the promoter encoding sequence since these regions of AvrPita, PWL1 and PWL2 are sufficient to direct fluorescently tagged proteins into the biotrophic interfacial complex (Khang et al. 2010). The role of the biotrophic interfacial complex and the mechanism underlying effector uptake across the host membrane has yet to be elucidated.

Passage of fungal and oomycete effectors into the plant cell cytoplasm is a crucial aspect of pathogenicity and elucidation of the mechanism of effector uptake is clearly an important step in understanding the infection process. In contrast to the Type III secretion system of Gram-negative bacteria (Cornelis 2006; Alfano and Collmer 2004; Galan and Wolf-Watz 2006) or the PEXEL protein export system of the *Plasmodium* malarial parasites (Boddey et al. 2010; de Koning-Ward et al. 2009), uptake of fungal and oomycete effectors does not require participation of any other pathogen-encoded molecules. Uptake of fungal and oomycete effectors depends solely on plant-encoded components.

Several mechanisms of pathogen-independent protein uptake into the cytosol of host cells have been proposed, as summarised in Fig. 7. These can be broadly categorised into (1) endocytic uptake, (2) transport through a transmembrane transporter and (3) direct membrane penetration (Drin et al. 2003; Goldberg and Cowman 2010; Voegele et al. 2009). After endocytosis, material may be released via retrotranslocation through the Golgi apparatus and endoplasmic reticulum (ER) into the cytoplasm (route 1a) or directly from the endosomes (route 1b). Retrotranslocation is normally associated with cellular protein quality control, a process in which misfolded proteins are transported from the ER into the cytosol where they are targeted for proteosomal degradation. However, some pathogen proteins, such as the Shiga and ricin toxins, are able to subvert this process for delivery into the host cytosol (Magzoub et al. 2005; Sandvig and van Deurs 1996; Sandvig and van Deurs 2002).

Multiple forms of endocytosis are known to occur in animal cells, with the main two mechanisms involving clathrin-mediated and lipid raft-associated pathways (Doherty and McMahon 2009; Mayor and Pagano 2007). Plant genomes include genes encoding a number of proteins that function in clathrin-mediated endocytosis (Barth and Holstein 2004; Holstein and Oliviusson 2005) and clathrin-mediated uptake has been shown to participate in endocytosis of the PIN auxin efflux carrier (Dhonukshe et al. 2007). During infection of broad bean by *U. fabae*, coated vesicles are seen in the plant cytoplasm surrounding the haustorium, suggesting that clathrin-dependent endocytosis is taking place (Xu and Mendgen 1994), however, whether or not endocytosis might be internalising *U. fabae* effectors is not known. Recent inhibitor studies did not give any evidence to suggest that uptake of the flax rust effector AvrL567 and the *P. infestans* effector Avr1b into human A549 cells requires clathrin-dependent receptor-mediated endocytosis (Kale et al. 2010). However, immunogold localisation of the flax rust effector AvrM in high pressure-frozen and freeze-substituted material does provide evidence of vesicle-mediated uptake of AvrM (P.H.P. Gan and A.R. Hardham, unpublished observations). In animal cells, some forms of lipid raft-associated endocytosis involve caveolin or flotillin proteins. Although there is no evidence of caveolin

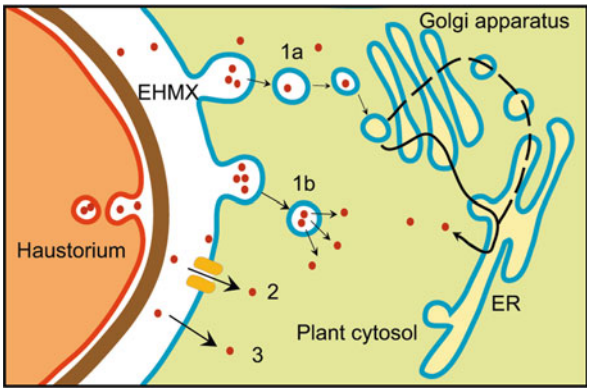

Fig. 7 Possible routes for effector entry. Effectors (*red dots*) may enter plant cells in the absence of the pathogen via endocytic uptake (1a + b), where escape from endosomes into the cytosol may occur via retrotranslocation through the Golgi apparatus and the endoplasmic reticulum (ER) (1a) or via endosomal escape (1b); transfer through a transmembrane transporter (2) and/or through direct membrane penetration (3). Figure design modified from Voegele et al. (2009). *EHMX* extrahaustorial matrix

in plant genomes (Aniento and Robinson 2005), flotillins have been localised to lipid rafts in the plasma membrane in *Arabidopsis* and *M. truncatula* (Haney and Long 2010; Borner et al. 2005). In *M. truncatula*, establishment of nodules by the bacteria *Sinorhizobium meliloti* during symbiosis requires two flotillins, possibly for the trafficking of signals co-ordinating early symbiotic events or regulating nodulation (Haney and Long 2010).

The possible role of lipid rafts in fungal and oomycete effector uptake has recently been explored as part of a study of effector binding to membrane lipids (Kale et al. 2010). The proposal arising from this investigation is that fungal and oomycete effector entry into host cells is mediated by binding to the lipid phosphatidylinositol-3-phosphate (PtdIns(3)P) on the extracellular leaflet of the lipid bilayer of the host plasma membrane (Kale et al. 2010). This study presents evidence that oomycete effectors bind to PtdIns(3)P via the RXLR motif and that this interaction is required for effector uptake (Kale et al. 2010). Phosphotidylinositides constitute a small but important group of phospholipids within cells. They are involved in signalling processes and contribute to spatial regulation of cellular organisation through enrichment of different subsets of phosphotidylinositides in specific organelle membranes or membrane domains (Heilmann 2009). Kale et al. (2010) suggest that other fungal effectors, including AvrL567 from *M. lini* and AvrLm6 and AvrLm2 from *L. maculans*, may also enter host cells by PtdIns(3)P-binding. However, although AvrM was observed to bind to PtdIns(3)P, studies of GST fusions of deletion AvrM mutants have shown that polypeptides that are taken up *in planta* do not necessarily exhibit PtdIns(3)P binding activity (Gan et al. 2010), indicating that PtdIns(3)P-binding is not required for AvrM entry into host cells.

Another fungal protein that internalises into plant cells in the absence of the pathogen is the PtrToxA toxin from the causal agent of wheat tan spot, *Pyrenophora tritici-repentis* (Manning and Ciuffetti 2005). Toxin entry is dependent on the presence of an RGD vitronectin-like motif (Manning et al. 2008). In animal cells, the RGD trimer mediates binding of extracellular matrix proteins to integrins in the plasma membrane, and integrins are known to be targets of mammalian pathogens which internalise into host cells via a form of clathrin-mediated uptake (Isberg and Tran Van Nhieu 1994). Uptake of PtrToxA occurs in ToxA-sensitive but not ToxA-insensitive host mesophyll cells and the protein accumulates close to the infiltration site, suggesting interaction with a high-affinity receptor on the surface of the host cell and internalisation via a form of receptor-mediated endocytosis (Manning and Ciuffetti 2005). The RGD motif is also present in the *P. infestans* IPI-O effector (which also contains an RXLR motif) and IPI-O has been shown to compete with other RGD-containing peptides in binding to the plant plasma membrane (Senchou et al. 2004). However, this motif is absent from the AvrM and AvrL567 minimum uptake regions identified by Rafiqi et al. (2010), indicating that binding to integrins may not be the mechanism of host cell entry for these fungal effectors.

Another category of proteins that are able to enter cells autonomously are those containing cell penetrating peptides (CPPs) (Frankel and Pabo 1988; Green and Loewenstein 1988; Joliot et al. 1991; Thorén et al. 2000). CPPs have highly divergent sequences although many are amphipathic or cationic in nature (Drin et al. 2003). CPPs can mediate uptake of attached cargoes, including low molecular weight drugs, proteins, nucleic acids and peptides (Rothbard et al. 2000; Nagahara et al. 1998; Simmons et al. 1997; Shibagaki and Udey 2002; Chugh and Eudes 2008). CPPs may be internalised by virtue of their secondary structure and net positive charge (Drin et al. 2001, 2003) and there is evidence that their internalisation can occur via different mechanisms including direct membrane penetration through pore or inverted micelle formation, clathrin-dependent endocytosis, lipid raft-mediated macropinocytosis or clathrin- and caveolin-independent endocytosis (Drin et al. 2003; Richard et al. 2005; Wadia et al. 2004; Ter-Avetisyan et al. 2009; Joliot and Prochiantz 2004). Like CPPs, the uptake domains of AvrM and AvrL567 have highly divergent sequences and it remains possible that uptake of these and other fungal effector proteins could depend on the secondary structure of the N-terminal motifs.

9 Summary

Micro-organisms have adapted to become efficient pathogens of plants, establishing growth even within living host cells through specialised infection structures such as haustoria and intracellular invasive hyphae. In biotrophic interactions where pathogens obtain nutrients from living hosts, pathogens secrete small proteins known as effectors, which contribute to the establishment and

maintenance of infection. While considerable progress has been made in identifying candidate effectors through high throughput screening of pathogen genome sequences, the functions of these proteins are only just beginning to be characterised. It is becoming clear that effectors have roles not only in the apoplast but also within host cells. Key areas for future research include elucidating the functions of effectors and characterising the mechanism of host intracellular fungal and oomycete effector delivery into host cells.

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Compatibility in Biotrophic Plant–Fungal Interactions: *Ustilago maydis* and Friends

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Abstract Biotrophic plant pathogens depend on living host cells during all stages of pathogenic interaction. *Ustilago maydis*, the causative agent of smut disease induces development of tumors on all aerial organs of its host plant maize. Immediately upon host penetration, biotrophy is established and maintained during fungal proliferation and nutrition up to the formation of sexual spores. This requires an efficient suppression of plant defense responses, in particular host cell death. In the molecular communication between pathogen and its host, secreted effector proteins play essential roles. The actual functions of these effectors, however, still remain largely elusive. To successfully execute the different steps of pathogenic interaction, a tight regulatory network has evolved in the pathogens, coordinating expression of secreted effectors in a stage- and organ-specific manner. In this chapter, we discuss the complex molecular mechanisms that ensure compatibility in the intimate relationship between biotrophic fungi and their plant hosts.

1 Introduction

During evolution biotrophic fungi developed a multiplicity of strategies to cope with their plant hosts. Mechanisms to establish compatible biotrophic interactions mainly involve the escape from host recognition as well as manipulation of the plant defense systems and reprogramming of its metabolism. In this chapter, we will highlight the key features of fungal biotrophic plant pathogens. The basidiomycete smut fungus *Ustilago maydis* that parasitizes on corn represents an important model organism for biotrophic plant–fungal interactions and will be discussed

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in detail. Particular emphasis will lie on the crucial role of secreted effectors in the crosstalk between the parasite and its host.

1.1 Definition of Biotrophic Plant–Parasite Interactions

One way to classify plant pathogens is according to their feeding strategy (Oliver and Ipcho 2004). By definition biotrophic plant parasites rely on living substrates. Many of them establish long-term feeding relationships with their hosts (Lewis 1973; Staples 2000; Mendgen and Hahn 2002). These characteristics are also resembled by the term biotrophy that has been derived from the Greek words *bios* ("life") and *trophy* ("feeding"). In contrast to biotrophs, necrotrophic pathogens (e.g., *Sclerotina sclerotioum*, *Botrytis cinerea*) developed a feeding strategy based on destroying of the host tissue to obtain food from the dead plant material (Lewis 1973). Therefore, necrotrophs kill host cells by secreted toxins which allow the parasites to grow saprophytically on the released nutrients (Farrar 1984; Walton 1996; van Kan 2006).

To succeed in their intimate host relationship, biotrophs have evolved strategies to keep the invaded plant cells alive during infection. The entire pathogenic development including adhesion, penetration, host colonization as well as the completion of the life cycle by spore formation needs to occur with minimal damage of the host. Major challenges of biotrophic pathogens to assure compatibility are therefore on one hand to mask themselves in order to circumvent recognition by the host and on the other hand, to suppress those host defense mechanisms that cannot be avoided (van Esse et al. 2007; de Wit 2007). Parasitic biotrophs obtain nutrients from shoot tissue, having no alternative energy sources. Thus, the host metabolism needs to be reprogrammed in a way that ensures a continuous supply of nutrients to the pathogen (Spanu and Kämper 2010).

Biotrophy is not restricted to a single taxonomic group of plant pathogens but exists in different kingdoms ranging from fungi (i.e., basidiomycetes like rusts and smuts; ascomycetes like powdery mildews) and oomycetes (i.e., downy mildews) to the bacteria (i.e., *Pseudomonas syringae*, *Xanthomonas campestris*). Even the animal kingdom is represented by plant-parasitizing root-knot and cyst nematodes. It is not yet clear whether biotrophy is a result of convergent evolution in the different taxonomic groups or if this lifestyle has a very early common evolutionary origin (Schulze-Lefert and Panstruga 2003). An interesting view on biotrophy was given by the finding that endophytic fungi hold the potential being pathogens. This was nicely shown in recent studies on the *Epichloë*/*Neotyphodium* endophytes that usually colonize rye grass in symbiotic interactions (Tanaka et al. 2006, 2008). In this light, the evolutionary question about emergence of biotrophy might be asked: are we dealing with a preliminary stage of development towards a mutualistic interaction, or might symbionts represent pathogens that are tamed by their respective host plants?

Biotrophic organisms comprise important plant pests that cause enormous economic losses of agricultural plants worldwide. Furthermore, in natural environments they can reduce the competitive abilities of the host. While economically not threatening biotrophs only lead to moderate agricultural problems, others are much more harmful pests. Prominent examples of devastating plant pathogenic fungal biotrophs are the cereal rusts like *Puccinia graminis* f. sp. *tritici* (Staples 2003). In the second half of the nineteenth century, the coffee bean rust *Hemileia vastatrix* caused devastating losses in Sri Lanka. This led to collapse of the coffee industry, which is the reason for this region mainly producing tea and rubber nowadays (Waller et al. 2007).

Biotrophic host–parasite relationships can be maintained either transiently or during the complete pathogenic program up to sporulation. Transient types of biotrophy that are referred to as hemibiotrophy are observed, e.g., in the fungi *Magnaporthe grisea*, in different *Colletotrichum* subspecies, and in the oomycete pest *Phytophthora infestans* (Latunde-Dada 2001; Mendgen and Hahn 2002; Hammond-Kosack and Parker 2003; Haldar et al. 2006). Here, the parasite initially establishes a biotrophic interaction with its host plant that switches to a necrotrophic stage while the infection proceeds (Perfect and Green 2001).

Upon the "true" biotrophs that stay biotrophic throughout their complete life cycle obligate and nonobligate parasites must be differentiated. Obligate biotrophs are in many cases difficult or not at all cultivable in the absence of their host plant, which causes immense experimental disadvantages in their study. On the other hand, nonobligate biotrophs like *U. maydis* are able to grow under artificial laboratory conditions and are amenable to genetic modifications. Therefore, they often deal as model systems for biotrophy (Brefort et al. 2009).

2 Pathogenic Development of Biotrophic Fungi

Although being capable of propagating saprophytically, nonobligate biotrophs rely on the biotrophic phase for sexual recombination. Most biotrophic fungi share a common infection program. First stages of pathogenic development include host recognition, attachment to the surface, and penetration at suitable surface sites. The differentiation of specialized infection structures termed appressoria is widely spread upon plant-pathogenic fungi. This structure mediates a direct penetration of the plant tissue, an important stage that resembles the switch from extracellular to invasive biotrophic growth. Therefore, the penetration event is considered to be a complex process. Some pathogenic fungi like the rice blast *M. grisea* or *Colletotrichum* spp. facilitate invasion of epidermal cells by mechanical force (Howard et al. 1991; Money and Howard 1996). In this case, penetration is promoted by an enormous turgor pressure that develops in the dome-shaped, melanized appressoria of these pathogens (i.e., 8 MPa in *M. grisea*; Tucker and Talbot 2001). Alternatively, host penetration may be mediated by the local secretion of lytic enzymes or by a combination of both mechanisms employing slightly lower turgor

pressures, i.e., as it has been observed in *Blumeria graminis* (Bailey et al. 1992; Francis et al. 1996; Pryce-Jones et al. 1999). In the latter cases, infection structures are usually less prominent and nonmelanized (Mendgen and Deising 1993).

Following penetration, colonization of the plant occurs in the host tissue. Although biotrophs all rely on living material during plant colonization, the actual relationship to the host is realized in multiple ways. Spreading in the plant can occur subcuticular like, e.g., observed in plant infections with the ascomycete *Venturia inaequalis*, causing apple scab disease. Some pathogens like *U. maydis*, *Claviceps purpurea,* and monokaryotic rust fungi spread inter- as well as intracellularly. Powdery mildews, dicaryotic rust fungi, and downy mildews grow mainly extra- or intercellular but develop specialized intracellular feeding structures which can be situated either in epidermal or parenchymal tissue (reviewed in Mendgen and Hahn 2002).

Although plant colonization proceeds in many different ways, a common principle of invasive growth is the invagination of the host plasma membrane, which creates a matrix-filled interface between the parasite and the host cell, termed the biotrophic interface (Bauer et al. 1997). This unique compartment is the site of communication between pathogen and host and thus, probably *the* most important scenery during the establishment of the biotrophic interaction. In the past years it has become more and more evident that proteins secreted to the interface are the mediators of parasite–host communication. Remarkably, a subset of the weaponry of secreted effectors the pathogen sends to the interaction zone even enters the plant cell where the proteins function either in the cytoplasm or in organelles like the nucleus (see Sect. 3; Kemen et al. 2005; Khang et al. 2010).

An additional characteristic feature of many (e.g., rust fungi, powdery mildews) but not all biotrophic fungi is the development of intracellular feeding structures, the so-called haustoria. Typically, haustorium-forming biotrophs grow extra- or intercellularly, invading only a few plant cells which are then completely filled with fungal material. Thus, a nutrient sink is generated at the infection site to ensure feeding of the parasite (Mendgen and Hahn 2002; O'Connell and Panstruga 2006). The extended haustorial surface area might provide a perfect prerequisite for nutrient flow towards the parasite. Some biotrophs like *U. maydis* do not differentiate haustoria (Brefort et al. 2009). Nevertheless, it has been demonstrated that those pathogens are also able to establish a nutritional sink tissue at the infection site (Doehlemann et al. 2008b; Horst et al. 2008), probably mediated by the biotrophic interfaces surrounding intracellular growing hyphae.

3 *Ustilago maydis* as a Model System for Biotrophic Fungi

U. maydis is the leading model organism of the plant pathogenic smut fungi. The basidiomycete fungus parasitizes only on *Zea mays* and its wild progenitor teosinte (*Z. mays* ssp. *mexicana* and ssp. *paviglumis*) where it causes corn smut disease. Concomitantly, *U. maydis* induces the formation of plant tumors in which the

fungus completes its disease cycle (Christensen 1963; Banuett 1995; Banuett and Herskowitz 1996; Doehlemann et al. 2008a).

U. maydis has a diphasic life cycle: the fungus grows saprophytically as haploid sporidia but cannot cause disease in this form. To infect maize plants, sporidia of different mating type need to recognize each other on the host surface via a pheromone–receptor system (Bölker et al. 1992). Perception of the compatible pheromone induces formation of conjugation tubes that fuse at their tips and thus form the infectious dicaryotic filament (Sleumer 1932; Rowell 1955; Snetselaar 1993; Snetselaar et al. 1996). This filament is able to enter the plant tissue directly, which is accompanied by swelling of the hyphal tip resulting in an appressorial structure (Snetselaar and Mims 1992). Immediately upon host invasion, the biotrophic relationship is established and fungal hyphae grow intracellularly in the infected host tissue, being surrounded by a biotrophic interface (Fig. 1).

Biotrophic hyhae proliferate massively, particularly in mesophyl and vascular tissue until basidiospore formation occurs (Snetselaar and Mims 1994; Doehlemann et al. 2008a). This goes along with formation of tumors on all areal parts of the infected maize plants (Brefort et al. 2009). The diploid spores are released when the tumors dry up and rupture. Under favorable conditions these spores germinate, the diploid nucleus undergoes meiosis, and budding off from a promycelium,

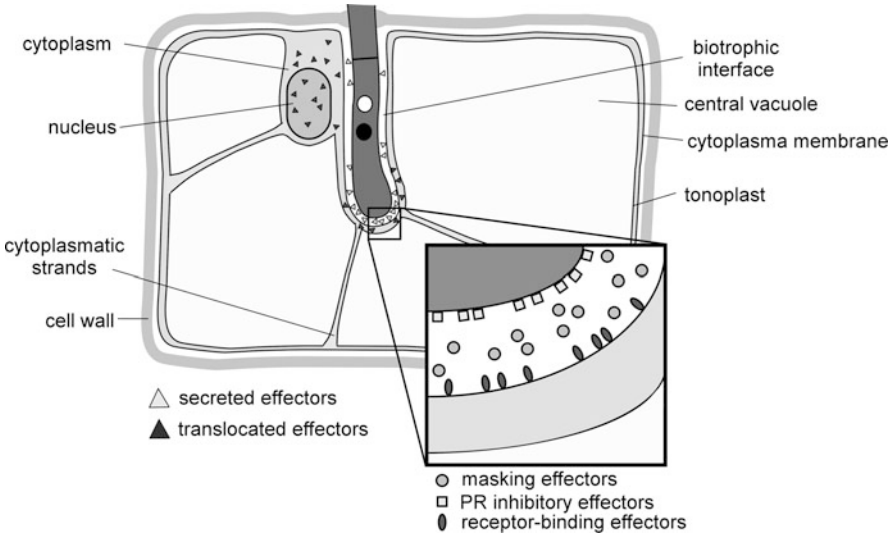

Fig. 1 Potential sites of *U. maydis* effector activity. The figure represents a schematic view of a maize cell that has been invaded by an intracellular growing fungal hypha. During this process, the plant plasma membrane invaginates and the fungal hypha is separated from the plant symplast by the biotrophic interface. Since adult maize cells contain a large central vacuole the cytoplasm containing cell organelles resembles up only a thin layer directly adjacent to the plant cell wall and the fungal hypha. Secreted effectors localize to the biotrophic interaction zone (*open triangles*). Moreover, effectors potentially enter the plant cell (*dark triangles*). The close up view visualizes three different putative functions of the secreted apoplastic effectors

haploid sporidia are again produced (Christensen 1963; Snetselaar and Mims 1994; Banuett 1995; Banuett and Herskowitz 1996; Kahmann et al. 2000).

The *U. maydis–Z. mays* pathosystem has become more and more important not only as a model for plant pathogenic smut fungi but also for one of the few models for "true" biotrophic host–pathogen interactions (Kämper et al. 2006; Brefort et al. 2009). In contrast to obligate biotrophs, haploid *U. maydis* cells can be efficiently grown in synthetic media and genetic modifications can be performed with a convenient toolbox of established methods available, i.e., an efficient gene replacement system, a set of constitutive and inducible promoters as well as genetically modified strains that grow filamentously in axenic culture (Kämper 2004; Spellig et al. 1996; Brachmann et al. 2001; Zarnack et al. 2006). In addition, advanced life cell imaging and a variety of staining methods have been established for *U. maydis* to characterize in planta development of the fungus (Basse and Steinberg 2004; Doehlemann et al. 2008a, b; Brefort et al. 2009).

The 20.5-Mb genome of *U. maydis* has been sequenced, manually refined and sequences are publicly available as well as regularly updated on a MIPS platform (http://www.broad.mit.edu/annotation/genome/ustilago_maydis/Home.html; http://mips.gsf.de/genre/proj/ustilago/; Kämper et al. 2006). Genome availability furthermore laid the foundation for the establishment of DNA microarray profiling (Kämper et al. 2006).

For *U. maydis* infection, sporidia of compatible mating types have to meet and fuse on the leaf surface, leading to the formation of a diploid infectious filament. The development of solopathogenic strains that do not need compatible mating partners has further improved the pathosystem allowing the fast generation and subsequent phenotyping of *U. maydis* mutants with no longer need to introduce mutations in two haploid wild-type strains (Bölker et al. 1995; Kämper et al. 2006).

Disease assays can be performed on young maize seedlings with development of symptoms (i.e., chlorosis, anthocyan, tumors) within two weeks after sowing (Banuett 1995). Standardized disease rating combined with confocal microscopic imaging techniques and PCR-based quantification of fungal biomass permits not only discrimination between infected or noninfected plants but also allows specification of symptom severity as well as the degree and manner of intra- and intercellular proliferation of the pathogen (Kämper et al. 2006; Doehlemann et al. 2008b; di Stasio et al. 2009). Thus, this system allows not only the identification of essential virulence factors but also of *U. maydis* mutants with slightly altered virulence ranging from minor reductions to hypervirulence (Kämper et al. 2006).

Genetic modifications of the host plant maize like gene silencing or gene insertions are possible though very time consuming (Shrawat and Lörz 2006). However, a systemic virus-induced gene silencing system for maize has recently been developed to study host genes during *U. maydis* infection. This tool will increase the methodological potential for future studies on this pathosystem (van der Linde et al. 2010).

4 Plant Responses to *U. maydis*

Plants have evolved multifaceted defense mechanisms against pathogen attacks. In a first line of defense, recognition of conserved microbial molecules (pathogen-associated molecular patterns, PAMPS) elicits a basal defense response characterized by the induction of pathogenesis-related (PR) genes, strengthening of cell walls as well as the production of secondary metabolites. Often, these responses are associated with the production of reactive oxygen species and the induction of localized cell death (the hypersensitive response, HR), leading to a PAMP-triggered resistance (Jones and Dangl 2006).

Specific virulence factors suppress PAMP-triggered responses. However, they again might be recognized by plant resistance genes that developed in a co-evolutionary arms race. Defense responses activated by resistance genes involve HR induction, associated with changes in phytohormone levels. A process leading to the so-called, effector-triggered immunity (Jones and Dangl 2006), The phytohormone changes are specifically adapted to the attacking pathogen. The classical, widely accepted model describes salicylic acid (SA)-dependent defenses acting against biotrophic pathogens. In contrast, jasmonate (JA)- as well as ethylene-dependent responses were shown to defend infections with necrotrophs (Greenberg and Yao 2004; Glazebrook 2005; Jones and Dangl 2006; O'Connell and Panstruga 2006).

In the compatible *U. maydis–Z. mays* pathosystem indications of a transient induction of plant defense responses can be recognized on the micro- and macroscopic level, which is changed upon successful establishment of biotrophy about 20–24 h after inoculation (Doehlemann et al. 2008b). During the early phase of plant colonization, intracellular *U. maydis* hyphae grow at the apex thereby moving their cytoplasm to the tip compartment. At the same time older hyphal parts remain empty and are sealed off by septae (Snetselaar and Mims 1994). Hyphal autofluorescence develops at the empty sections, indicating enhanced recognition of these old parts by the plant. Finally, plant cells containing such collapsed hyphae undergo cell death (Doehlemann et al. 2008b). These processes coincide with early infection symptoms which are chlorosis and, eventually, small necrotic spots that develop at the infection sites and which are most likely caused by single dying plant cells.

Later on, in colonized tissues, strong autofluorescence and occasional small areas with clusters of dead cells were detected, indicating enhanced plant defense sporadically elicited by some hyphae (Doehlemann et al. 2008b). Finally, *U. maydis*-induced tumors are formed by enlargement as well as proliferation of plant cells which goes along with massive proliferation of the fungus. Although large fungal aggregates are formed in intercellular spaces, programmed cell death (PCD) can only rarely be observed in the surrounding plant tissue (Doehlemann et al. 2008a, b).

To investigate plant reactions on the molecular level, transcriptional changes of maize in response to *U. maydis* infection were studied by a differential display approach (Basse 2005) as well as by a time-resolving analysis using the Affymetrix DNA microarray system (Doehlemann et al. 2008b). Microarray analyses revealed

that an initial recognition of the fungus takes place on the leaf surface, eliciting a strong but rather unspecific plant defense response. The transient induction of plant defense genes suggests that *U. maydis* is recognized via PAMPs. In accordance with this hypothesis, maize orthologs of specific PAMP-induced LRR-like receptor-like kinases were shown to be transcriptionally induced during this early phase of infection (Doehlemann et al. 2008b; Zipfel et al. 2004, 2006). Additionally, a SER kinase belonging to a group that includes BAK1 was up-regulated (Doehlemann et al. 2008b). BAK1 has been shown to act as a positive regulator in infection-induced cell death signaling (Chinchilla et al. 2007; Kemmerling et al. 2007). These observations indicate that PAMP-induced signaling is indeed activated during the penetration phase of *U. maydis* resulting in a strong and unspecific plant response.

However, with establishment of the biotrophic interaction 24 h after infection, the initial unspecific defense responses were attenuated (Doehlemann et al. 2008b). Evidence for this was provided by the observation that previously induced defense gene expression decreased significantly with the onset of biotrophy. In parallel, induction of genes encoding cell death suppressors such as Bax-Inhibitor-1 (Eichmann et al. 2004) as well as the repression of caspases was observed (Doehlemann et al. 2008b). These transcriptional changes ensure the secondary suppression of the initially elicited unspecific defense mechanisms which is a prerequisite for establishment of biotrophy.

The specific plant response to *U. maydis* infection is also mirrored by transcriptional changes in plant hormone signaling. During compatible interactions, biotrophic pathogens induce primarily JA and ethylene responses. These hormones act antagonistic to SA signaling which counteracts biotrophic pathogens and are associated with induction of tryptophane biosynthesis, the accumulation of secondary metabolites, and the induction of plant genes encoding defensins (Brader et al. 2001; Glazebrook 2005; Wasternack 2007). In line with this, an induction of JA signaling as well as typical JA-responsive defense genes such as defensins and Bowman–Birk-like proteinase inhibitors was also observed during *U. maydis* infections (Basse 2005; Doehlemann et al. 2008b). Moreover, transcripts of PR1, a marker protein for SA-dependent cell death responses could not be detected during the early biotrophic phase of *U. maydis* infection, indicating repressed SA signaling (Seo et al. 2001; Doehlemann et al. 2008b). In summary, these studies suggest that, once a biotrophic interface is established, *U. maydis* suppresses defense responses and in particular HR-induced cell death.

Besides its ability to suppress defense mechanisms of the host plant a second process that characterizes the biotrophic *U. maydis* infection is the reprogramming of the plant physiology. So far detailed metabolic studies revealed a redirection of carbohydrate and nitrogen fluxes towards the infected tissue in which massive proliferation of fungal hyphae occurs (Horst et al. 2008; Doehlemann et al. 2008b; Horst et al. 2010).

Measurements of hexoses and hexose/sucrose ratios revealed that about 4–6 days post infection infected leaf areas convert from a carbon *source* to a *sink* tissue (Billet and Burnett 1978; Doehlemann et al. 2008b; Horst et al. 2008). In line

with this, transitory starch production and sucrose accumulation during the light period were dampened in tumor tissue.

Maize is a C_4 plant. However, in the immature stage seedlings still exhibit C_3 or C_3–C_4 intermediate photosynthesis (Nelson and Dengler 1992; Langdale and Kidner 1994). During early leaf development, uninfected juvenile maize seedlings undergo a transition to C_4 metabolism. In contrast, characteristic features of C_3 photosynthesis are kept throughout *U. maydis* infections of maize seedlings, demonstrating that the transition to C_4 metabolism is prevented in infected seedlings (Horst et al. 2008; Doehlemann et al. 2008b). *U. maydis* does not infect differentiated source leaf tissue (Wenzler and Meins 1987). This suggests that during infections of immature seedlings this fungus reprograms the developing tissue in a way that the sink status is retained rather than transforming source leaves back to sink leaves.

Stable isotope probing experiments demonstrated that *U. maydis* tumors furthermore constitute strong nitrogen sinks that rely on organic nitrogen import from noninfected, systemic source leaves (Horst et al. 2010). In accordance with this, Horst et al. (2010) provided evidence that the systemic source leaves display an enhanced photosynthetic capacity as well as an increased amino acid export. These amino acids are transported via the phloem to the tumor tissue where they are metabolized to nitrogen-rich amino acids. These data suggest that the increased import of nitrogen into tumor tissue might feed the protein biosynthesis of *U. maydis* (Horst et al. 2010).

Recently, the identification of a novel sucrose transporter essential for pathogenicity improved our understanding of how *U. maydis* extracts nutrients from its host (Wahl et al. 2010a). This specific transporter allows the direct utilization of sucrose at the plant–fungal interface without extracellular hydrolysis and, thus, avoids the production of extracellular monosaccharides known to elicit plant immune responses (Wahl et al. 2010a). This finding also provides one example of the means *U. maydis* employs to prevent the excessive elicitation of plant responses during biotrophy.

5 Surface Sensing and Penetration

Initiation of different prepenetration stages on the plant surface is a prerequisite for the successful establishment of biotrophy. These stages involve cell attachment, the exploitation of suitable penetration sites, and the subsequent differentiation of penetration structures. This requires a complex molecular dialogue between fungus and plant (Dixon and Lamb 1990; Apoga et al. 2004).

On the plant surface, *U. maydis* undergoes a dimorphic switch from budding to hyphal growth. Once compatible haploid sporidia recognize each other via a pheromone–receptor system they form conjugation tubes that fuse at their tips. This generates a dicaryotic filament – the infections form of *U. maydis* (Sleumer 1932; Bowman 1946; Rowell 1955; Snetselaar and Mims 1992; Snetselaar 1993;

Spellig et al. 1994). Attachment of *U. maydis* filaments to the hydrophobic plant surface is likely mediated by repellent peptides. The *rep1* gene encodes a secreted precursor protein that is proteolytically cleaved into 11 peptides with high sequence similarity during secretion (Wösten et al. 1996; Teertstra et al. 2006). These peptides have functionally replaced hydrophobins in *U. maydis* and are likely to function as amyloid-like structures on the hyphal surface (Teertstra et al. 2009).

About 16 h post infection fungal filaments growing on the plant surface differentiate appressoria that enable the fungus to enter the plant tissue and therefore, initiate the switch to invasive growth (Snetselaar and Mims 1992; Mendoza-Mendoza et al. 2009). The differentiation of filaments as well as appressoria in *U. maydis* is dependent on host-derived physicochemical cues that are perceived by the fungus (Mendoza-Mendoza et al. 2009).

The plant cuticle consists of two layers. The uppermost layer which is in direct contact with the environment is a continuous layer of epicuticular waxes. The second, inner layer is composed of intracuticular waxes associated with a polyester matrix of cutin (Eigenbrode and Espelie 1995; Kolattukudy 2001). Cutin itself resembles a network of interesterified hydroxyl and epoxy derivatives of C_{16} and C_{18} fatty acids (Purdy and Kolattukudy 1975). Mendoza-Mendoza et al. (2009) demonstrated that hydrophobicity induces the formation of dicaryotic *U. maydis* filaments on artificial surfaces that resemble those growing on the surface of infected plants. In the natural environment, this hydrophobic surface is most likely provided by the plant cuticle.

An additional chemical cue, i.e., addition of hydroxy fatty acids, further stimulates filament formation and moreover, induces appressorium differentiation in the in vitro system. In addition, cutin monomers induce differentiation of appressoria at similar rates as observed for hydroxy fatty acids. It has been postulated that filamentously growing cells on the plant surface locally degrade plant cutin by the secretion of lytic enzymes leading to the release of cutin monomers. This may provide the stimulus that induces the differentiation of appressoria (Mendoza-Mendoza et al. 2009).

U. maydis appressoria are nonmelanized and are therefore unlikely to function through mechanical turgor force (Snetselaar and Mims 1993; Brachmann et al. 2003). This hypothesis is supported by the finding that during penetration, the cytoplasm can be located in the hyphal part that is still on the leaf surface as well as in the hyphal peg that already has penetrated (Schirawski et al. 2005).

Rather than employing mechanical force, *U. maydis* synthesizes a minimal set of secreted lytic enzymes that enables the fungus to penetrate (Mueller et al. 2008; Doehlemann et al. 2008a). Four secreted cutinases might degrade the cuticle that covers the leaf surface (Mueller et al. 2008). Beneath the cuticle the maize cell wall consists of glycan-crosslinked cellulose (Carpita et al. 2001; Abedon et al. 2006). Penetration of this rigid barrier is likely mediated by the rather limited set of plant-polysaccharide degrading enzymes which partially degrade and soften the plant cell wall to allow entry of the infectious filament (Mueller et al. 2008; Doehlemann et al. 2008a; Kämper et al. 2006). At the same time, the poor equipment with cell

wall degrading enzymes might prevent the excessive generation of molecules that lead to a stimulation of the plant defense machinery.

In contrast to the evident dome-shaped, melanized appressoria of pathogens like *M. grisea* the corresponding nonmelanized structures in *U. maydis* are rather hard to distinguish from other morphological structures. Characteristic but hardly recognizable features of filaments that form appressoria are slightly swollen tips which directly emerge from hyphal bends. Nevertheless, the recent establishment of an appressorial marker that consists of a *gfp* reporter gene fused to a promoter that is specifically induced in hyphal tip cells differentiating appressoria provides a sophisticated method to characterize differentiation and function of penetration structures in *U. maydis* (Mendoza-Mendoza et al. 2009).

6 Effectors

Plants employ a basal PAMP-induced defense system that recognizes pathogen attacks and subsequently triggers a defense program that includes an HR. Since an HR finally results in the PCD of the attacked cell this comprises a highly efficient way to block biotrophic pathogens. In the need to overcome this mechanism, pathogens developed specialized secreted effectors that counteract plant defense responses and thereby ensure compatibility (Jones and Dangl 2006).

A vast array of effectors has been identified in the past years of research. The described proteins show a high structural and functional variety. However, these proteins usually do not classify into known functional categories, i.e., enzyme classes but rather represent completely novel proteins with no homologies to proteins with described functions. Moreover, the secreted effectors often lack known functional domains. Therefore, research on these unique proteins is particularly challenging. Especially, detailed functional studies of fungal effectors are still scarce.

The question of effector function in biotrophic interactions is tightly intertwined with the localization of these proteins. As classical effectors contain an N-terminal signal peptide they are targeted to the secretory pathway of the pathogen and thus, end up in the biotrophic interface after secretion (Fig. 1). Three main scenarios of effector function were described until now: A variety of effector proteins inhibits plant PR proteins (Tian et al. 2004; Rooney et al. 2005; Fig. 1). Such function could, e.g., be assigned to the Avr2 effector of *Cladosporium fulvum*. This effector of the causal agent of tomato leaf mold is able to inhibit cysteine proteases of the host tomato (Rooney et al. 2005; van Esse et al. 2008). The hemibiotrophic oomycete *P. infestans* also secretes effectors that guard the pathogen through the inhibition of host proteases (Tian et al. 2004, 2007). *U. maydis* secretes a peroxidase that is likely to detoxify H_2O_2 during the initial phase of plant infection (Molina and Kahmann 2007). Moreover, secreted effectors can mask the pathogen and thereby prevent recognition by the host cell (Fig. 1). The chitin-binding virulence factor Avr4 of *C. fulvum* covers the fungal cell wall during plant infection and thereby, impedes the

action of chitinases (Westerink et al. 2002; van den Burg et al. 2006; van Esse et al. 2007). Recently, the LysM-domain containing effector Ecp6 of *C. fulvum* has been shown to prevent chitin-triggered immunity by sequestering chitin oligomers that are released from the cell walls of invading hyphae (de Jonge et al. 2010). Another reasonable function of pathogen effectors might be mediated by the binding to plant receptors that, e.g., trigger defense responses (Fig. 1). Hints for such functions are described interactions of secreted effectors with membrane-bound resistance proteins. In many cases these interactions are indirect, following the guard hypothesis (Dangl and Jones 2001; van der Hoorn and Kamoun 2008) – in other cases direct effector–receptor interactions have been demonstrated. An example is the *C. vulvum* Avr4 protein, which directly binds to the cognate Cf-4 receptor in tomato (van Esse et al. 2007).

In the last years it became more and more evident that secreted effectors of (hemi)-biotrophic pathogens not only reside in the biotrophic interface. Intriguingly, some effectors can enter plant cells (Fig. 1) where they usually directly interfere with defense-related processes (Ellis et al. 2009; Hogenhout et al. 2009; Stergiopoulos and de Wit 2009). A multiplicity of translocated pathogen effectors has been identified in phytopathogenic bacteria like *X. campestris* as well as in oomycetes. While the bacterial pathogens use a type III or IV secretion system to directly inject their effectors into the host cell a specific translocation motif (RXLR) has been identified in the N-terminal region of transferred oomycete effector molecules (Lahaye and Bonas 2001; Axtell et al. 2003; Gosh 2004; Kamoun 2007; Morgan and Kamoun 2007; Whisson et al. 2007). For phytopathogenic fungi, translocation into the plant cell has been shown for the *M. grisea* effector AVR-Pita, *Uromyces fabae* RTP1, and the flax rust effectors AvrM, AvrL657, AvrP123, and AvrP4 (Jia et al. 2000; Kemen et al. 2005; Catanzariti et al. 2006; Dodds et al. 2006; Rafiqi et al. 2010).

Plants potentially develop specific resistance (R) proteins that recognize pathogen effectors that interfere with defense responses. This in turn leads to effector-triggered immunity that is often associated with PCD induction (Jones and Dangl 2006). Remarkably in the *U. maydis*–maize interaction, such gene-for-gene systems, i.e., effectors that are specifically recognized by cognate resistance genes of the plant, have not been described so far. Possibly such an interaction has not been developed in this pathosystem, probably because the pathogenic pressure of *U. maydis* is not sufficient. An alternative explanation is that resistance against *U. maydis* has not been observed in maize plants because a comprehensive screen for resistance loci has not been performed yet.

In the recent past, the first effector proteins that actively interfere with PCD induction have been described in plant pathogenic bacteria and oomycetes. One example is the effector protein AvrPtoB of the plant pathogenic bacterium *Pseudomonas syringae* that is translocated into host cells (Rosebrock et al. 2007). The oomycete *Phytophtora infestans* encodes AVR3a which has been shown to suppress cell death in *Nicotinia benthamiana* (Bos et al. 2006); the *Phytophtora sojae* effector Avr1b facilitates suppression of BAX-induced cell death via action of

conserved motifs (Dou et al. 2008). For fungal effectors, a direct interference with PCD induction could not be proven so far.

Sequencing of the *U. maydis* genome revealed a set of 386 genes encoding novel proteins that are predicted to be secreted. Another 158 genes were predicted to code for secreted enzymes (Mueller et al. 2008). Proteins of the first class are likely to resemble secreted effectors that may have specific functions during biotrophic development of *U. maydis*. Remarkably, many of the genes for novel secreted proteins are arranged in clusters containing up to 24 genes (Kämper et al. 2006). During biotrophic development, the majority of these clustered, putative effector genes are transcriptionally up-regulated (Kämper et al. 2006). Twelve of these gene clusters were deleted in the solopathogenic *U. maydis* strain SG200 and five of the resulting mutants were significantly altered in virulence. While mutants of four gene clusters were attenuated in virulence and displayed defects at different stages of pathogenic development, one cluster mutant showed an increased virulence (Kämper et al. 2006). In addition to genes encoding secreted proteins that are arranged in clusters, *U. maydis* also comprises gene families where the individual genes are dispersed upon the different chromosomes. Recently, the development of a FLP-mediated marker recycling system enabled the analysis of such gene families in *U. maydis*. The complete deletion of an 11 gene family (*eff11*) resulted in a significant reduction of virulence (Khrunyk et al. 2010). This attenuation could be attributed to three of the genes. Another family of effectors is encoded by the *mig2* genes that were identified because of their strong up-regulation during plant colonization (Basse et al. 2002). Five of the *mig2* genes are clustered and a sixth gene is located independently on the genome (Basse et al. 2002; Farfsing et al. 2005). All *mig2* encoded proteins contain eight conserved cysteine residues, as it also has been found in Avr proteins of *C. fulvum* (Stergiopoulos and de Wit 2009). However, a mutant in which all six *mig2* genes were deleted showed no reduction in virulence when infected in maize seedlings. Given the high expression levels of these genes, one might speculate on putative functions of these effectors. So far, the mutants have only been tested in seedling infections of the maize variety Early Golden Bantam. Therefore, one possibility is that the function of the Mig2 protein family in virulence of *U. maydis* depends on the maize variety or the plant organ being infected. Particularly, a tissue-specific function of these proteins would be of interest, as recent findings suggest a similar situation for other *U. maydis* effectors as well (see Sect. 8; Skibbe et al. 2010).

A single secreted effector that is not arranged in a gene cluster encoding other secreted proteins has recently been shown to be essential for establishment of the biotrophic interaction of *U. maydis* with maize plants. This protein, termed Pep1 (*P*rotein *e*ssential during *p*enetration 1), is dispensable for saprophytic growth of *U. maydis* but deletion mutants are completely blocked in biotrophic development (Doehlemann et al. 2009). Confocal microscopy showed the mutant being able to penetrate the plant cell wall but subsequent invasion of the host cell stops immediately after invagination of the plant plasma membrane. At the same time, the mutant induces strong plant defense responses including PCD of attacked epidermis cells. Using fluorescently tagged versions as well as immunolocalization, the protein was

shown to be secreted from intracellular hyphae into the biotrophic interface, particularly accumulating when hyphae invade a so far uncolonized cell. However, the molecular function of Pep1 remains unclear. Since a Pep1 homolog of the barley covered smut fungus *Ustilago hordei* was able to complement *U. maydis* deletion mutants it is likely that it holds a general, conserved mechanism that is essential for pathogenicity of smuts (Doehlemann et al. 2009). Currently, biochemical approaches are applied to determine the molecular mechanism of Pep1. Given the fundamental role of this effector for pathogenicity, its plant targets are supposed to constitute major resistance factors of monocotyledonous plants to biotrophic fungi.

7 Regulation of Biotrophic Development

During biotrophic development, *U. maydis* undergoes a variety of stages that need to be tightly regulated to assure compatibility. Characterization of the underlying molecular mechanisms is essential to improve our understanding of how the fungus is able to succeed in the intimate relationship with its host plant.

The initial step during pathogenic development of *U. maydis* is the mating of two compatible sporidia on the plant surface (Bölker et al. 1992; Spellig et al. 1994). Cell fusion leads to the formation of an active transcription factor termed bE/bW, an heterodimeric protein that resembles a crucial cell cycle regulator. The active heterodimer induces a G2 cell cycle arrest that is maintained during filamentous growth on the leaf, during appressorium differentiation and during penetration of the host tissue. Directly after penetration, as soon as a compatible biotrophic interaction has been established, the G2 cell cycle arrest is released and filaments start to proliferate intra- and intercellularly in the host tissue (García-Muse et al. 2003; Scherer et al. 2006; Cánovas and Pérez-Martín 2009). *bE/bW* expression is a prerequisite for the transition from saprophytic to biotrophic growth *of U. maydis* since the b-heterodimer is required and sufficient to initiate pathogenic development and sexual reproduction (Bölker et al. 1995).

The essential role of the bE/bW transcription factor for pathogenic development could be characterized using a strain harboring a mutant allele that encodes a temperature-sensitive bE protein. This protein is instable at restrictive temperature. Plant pathogenicity assays under these conditions revealed a complete block of pathogenic development due to a failure in colonization of the host plant (Wahl et al. 2010b). Detailed microscopic analyses demonstrated that in planta hyphae develop severely enlarged, bulbous hyphal tip cells containing multiple nuclei, indicating a defect in cell cycle control and cytokinesis (Wahl et al. 2010b). Furthermore, transcriptional profiling under restrictive conditions conducted by DNA microarrays revealed a down-regulation of genes encoding secreted effectors during in planta growth. Intriguingly, the expression of 12 genes located in five *U. maydis* gene clusters encoding secreted effectors depends on *b*-activation and 10 of these belong to clusters that have been proven to execute important functions during pathogenicity (Wahl et al. 2010b; Kämper et al. 2006; see Sect. 3). These results

further demonstrated the importance of the bE/bW heterodimer as the central regulator of pathogenic development: The transcription factor is not only crucial for the establishment of the dikaryon and for initiation of biotrophy but also for its sustainment until sexual reproduction is completed (Wahl et al. 2010b).

To detect genes that are directly influenced by the bE/bW transcription factor with respect to their regulation the *b*-genes were hooked up to inducible promoters. Shifting of the *U. maydis* from restrictive to inductive growth conditions in axenic culture leads to a synchronized synthesis of the b-heterodimier. This allows comprehensive view on b-regulated gene expression by DNA microarray analyses (Heimel et al. 2010a). To this end, 347 b-regulated genes were identified, with a subset of 212 genes up- and the remaining 135 genes down-regulated (Heimel et al. 2010a). In sum, transcripts encoding proteins that are involved in cell wall remodeling, lipid metabolism, cell cycle control, mitosis, and DNA replication were up-regulated. Moreover, a transcriptional induction could be detected for genes that code for putative secreted effector proteins (Heimel et al. 2010a). Interestingly, only 14 of the 345 b-dependently regulated genes that have been identified by this transcriptional profiling monitoring *b* induction in axenic culture were also differentially expressed after temperature restriction of b activity in planta. This suggests that additional plant-specific signals and regulators modify b-mediated transcription (Wahl et al. 2010b).

One example for a directly b-activated gene that plays an important role during pathogenic development is Clp1. A Clp1 homolog has been shown to be essential for clamp cell formation in *Coprinus cinereus* (Inada et al. 2001). *U. maydis clp1* deletion strains are neither disturbed in appressorium formation nor in appressorium function. However, after penetration hyphal growth is arrested prior to the first mitotic division (Scherer et al. 2006). This indicates that *clp1* is required for proliferation in the host tissue. Further investigations revealed that *clp1* deletion leads to a failure in the formation of clamp-like structures during invasive growth as it has been described for the deletion of *clp1* in *C. cinereus*. As a consequence, in planta hyphae of *clp1* deletion mutants display a disturbed nuclear distribution (Scherer et al. 2006). While overexpression of *clp1* in haploid *U. maydis* strains does not lead to any obvious defects, overexpression of *clp1* in strains with an active bE/bW heterodimer strongly attenuates filamentation. Most likely, this observation is due to the fact that Clp1 counteracts the function of the bE/bW heterodimer. This is likely to occur via protein–protein interactions since the expression of the *b* genes is not affected by *clp1* expression. In line with this hypothesis it could be shown that a set of b-induced genes is down-regulated in *clp1* overexpressor strains (Scherer et al. 2006). Presumably, a Clp1-mediated transient inhibition of the bE/bW complex is needed to facilitate cell cycle progression during hyphal growth (Scherer et al. 2006).

Remarkably, most of the genes that were found to be transcriptionally controlled by the active bE/bW heterodimer do not possess putative bE/bW-binding sites in their promoter regions. Instead, it has been shown that a limited set of directly *b*-induced (so-called class I *b* targets) transcription factors are responsible for the regulation of the majority of *b*-regulated genes (90%; Heimel et al. 2010a). Thus,

the bE/bW protein does not directly influence the expression of all 347 target genes but rather triggers a complex multilayered regulatory network.

One particular class I target gene involved in regulation of downstream genes, the transcriptional regulator Rbf1, constitutes a C_2H_2 zinc finger protein. This transcription factor resembles the master regulator within the network of *b*-controlled genes during pathogenic development (Heimel et al. 2010b). Strains deleted in the *rbf1* allele display a similar phenotype as strains lacking an active bE/bW complex. Complementary, induction of *rbf1* leads to filament formation as well as a G2 cell cycle arrest, as it is also observed after activation of the bE/bW heterodimer (Heimel et al. 2010b).

The class I transcription factor Rbf1 controls another subset of regulators, the class II transcription factors. Two of them, Biz1 and Hdp1, are important during biotrophy (Heimel et al. 2010b; Spanu and Kämper 2010). Interestingly, Biz1 seems to regulate distinct stages of pathogenic development: The protein is dispensable for mating and filamentation but *biz1* deletion strains are strongly disturbed in appressoria formation and in planta proliferation. Only few hyphae are able to penetrate the plant epidermis and subsequent hyphal growth is stalled directly (Flor-Parra et al. 2006). Although *biz1* deletion strains display a very early pathogenicity defect, the protein might be essential during the complete pathogenic development since *biz1* expression is up-regulated during all stages of biotrophy (Flor-Parra et al. 2006).

Biz1 represses the expression of the cyclin *clb1*, resulting in a G2 arrest. Based on this it was speculated that the Biz1-induced cell cycle arrest is important though probably not sufficient for appressorium formation (Flor-Parra et al. 2006). The fact that *biz1* is highly expressed during the entire infection process might indicate that *U. maydis* cells growing within the plant tissue continuously need to go through cell cycle arrest stages.

U. maydis strains where genes for secreted effectors were deleted did not only display phenotypes that are due to an early block of biotrophic development. Some effector mutants showed reduced virulence and other rather subtle phenotypes that can be traced back to defects during later stages of plant colonization (Kämper et al. 2006). On these grounds it has been speculated that gene expression during all stages of pathogenicity is subject to a tight spatial and temporal regulation. Thus, there is a need for regulatory transcription factors that specifically control in planta gene expression. Recently, one such transcription factor, Mzr1, could be identified in a combined approach of promoter analysis and expression profiling (Zheng et al. 2008). This zinc-finger transcription factor controls, e.g., the in planta expression of three well-described genes that are specifically up-regulated during maize colonization. The three genes are part of the so-called *mig2*-gene cluster that consists of five related genes (*mig2-1* to *mig2-5*) coding for secreted proteins with yet unknown functions (Basse et al. 2002). Moreover, it has been demonstrated that the promoter region of one cluster gene, *mig2-5*, contains a *cis*-active element that mediates a strong transcriptional activation by Mzr1 during biotrophic development (Farfsing et al. 2005; Zheng et al. 2008). Although DNA microarray analyses revealed around 50 Mzr1-induced *U. maydis* transcripts with an enrichment of transcripts encoding

secreted proteins, deletion of *mzr1* only leads to reduced pathogenicity (Zheng et al. 2008). This result demonstrates again that regulation of pathogenic development cannot be traced back to a single transcription factor but that biotrophy is controlled by a complex network of transcription factors. Moreover, these transcriptional regulators seem to be tightly cross-linked as illustrated by the example of the interdependence between Mzr1 and Biz1: Although Mzr1 itself is independent from Biz1, activity of Biz1 was found to be necessary for expression of the *mig2–6* gene, which in turn is directly depending on Mzr1.

Another example of biotrophy-specific transcription factors in *U. maydis* is the forkhead protein Fox1. This protein is the first example of a *b*-independent transcription factor, indicating that biotrophic development is not only regulated by the *b*-network but also includes other important factors (Zahiri et al. 2010). Plants infected with *fox1* deletion mutants display enhanced defense reactions and therefore show drastically reduced virulence. This proofs the impact of *b*-independent regulators in *U. maydis* (Zahiri et al. 2010). Transcriptional profiling revealed about 130 *U. maydis* genes lacking transcriptional induction in the *fox1* deletion strain and these were again enriched for genes that code for putative secreted effectors (33 genes). However, single-gene deletion mutants for some of the most down-regulated *U. maydis* genes did not result in reduced virulence like observed for *fox1* deletion mutants. Thus, it may be speculated that only the simultaneous deletion of several or a distinct combination of Fox1-induced genes may mirror the *fox1* phenotype (Zahiri et al. 2010).

8 Stage and Organ Specificity of *U. maydis* Effectors

U. maydis virulence underlies complex regulatory networks during disease progression. However, it has recently been demonstrated for the first time that for establishment of biotrophy spatial regulatory fine tuning of infection seems to be of equal importance (Skibbe et al. 2010).

From the time when penetration occurs, *U. maydis* actively suppresses basal PAMP-triggered plant immune responses (Doehlemann et al. 2008b; see Sect. 4). However, biotrophy also includes active long-term manipulation of the host. Thus, it is not sufficient for the fungus to suppress only early plant defense responses. Secreted effectors which are known to be crucial for biotrophy are likely deployed for both processes. In accordance with this, there is experimental evidence that Pep1, an effector that is a major player in suppression of plant defense responses, is not only essential for the initial penetration of the plant surface but also for subsequent cell-to-cell passages in the maize tissue (Doehlemann et al. 2009).

Along with the analysis of gene clusters for secreted effectors in *U. maydis* also cluster deletion mutants with "mild" phenotypes due to defects during later stages of infection were identified. Observed phenotypes of cluster deletion mutants that did not completely loose virulence range from slight to strong reductions in tumor size and number to hypervirulence (Kämper et al. 2006). Though mutants like, e.g., the

cluster 19a deletion mutant are able to invade the plant tissue, tumor induction can be observed only very rarely (Kämper et al. 2006). These diverse phenotypes implicate a stage-specific activity of some secreted effectors. It seems obvious that *U. maydis* needs to employ specific sets of effectors during the different phases of infection since the requirements on the host will change with the course of infection that involves penetration, intracellular growth, and massive proliferation in intercellular spaces. The probably most elementary host cell reprogramming events need to occur during tumor formation – a process that is rarely understood to date. Unraveling the molecular mechanisms underlying these manipulations of the host by *U. maydis* effectors will be one particularly exciting aspect of future studies.

A unique feature of *U. maydis* in comparison to other, closely related smut fungi is its ability to induce tumor and subsequent spore formation in basically all aerial parts of its host plant. In contrast, symptom formation in infections with other smut fungi like, for instance, the head smut *Sporisorium reilianum* is restricted to the inflorescences of the respective host plants. With its ability to colonize a variety of maize tissues encountering cells with dissimilar grades of differentiation, *U. maydis* must be able to tailor its effector weaponry to these specific conditions (Skibbe et al. 2010). During ontogeny as well as in the mature form, each maize tissue produces a particular combination of proteins. Thus, it seems reasonable that multiple maize quantitative trait loci were identified which modulate the extent of tumor development in specific organs like the tassel, stalk, or ear tissue during *U. maydis* infection (Baumgarten et al. 2007).

With a simultaneous transcriptional profiling of *U. maydis* and *Z. mays* genes during the biotrophic stage, huge differences in the transcriptome of different infected host organs (seedling leaf, adult leaf, and tassel) were identified. The results indicated that the transition of leaf tissue to the convenience of the fungus requires significantly more changes in gene expression than in the tassel. Interestingly, not only the total number of regulated transcripts but also the respective functions of plant genes differentially expressed during *U. maydis* infection diverged between individual host organs (Skibbe et al. 2010). The results indicate that the special attribute of *U. maydis* to form tumors not only in the flowers but also on leaves must have been developed secondarily. In contrast, other smut fungi did not gain this competency. Moreover, the study proofed that mutants of the gene clusters encoding secreted effectors also display organ-specific phenotypes. The most intriguing example for this specificity is the cluster 19a deletion mutant: The corresponding strains induce tumor formation only very rarely in seedling leaves where the cells showed huge transcriptional changes during tumor induction. Contrarily, cluster 19a mutants behave almost like wild-type strains when tassels are infected, suggesting that the corresponding effectors are not crucial for symptom development in the male inflorescence (Skibbe et al. 2010). It will be a future challenge to elucidate which functions tissue-specific virulence factors exhibit during transformation of normal maize cell fates according to the specific requirements of the fungus in the respective organ of the plant.

According to the state of our knowledge, we conclude that the biotrophic interaction between *U. maydis* and its host *Z. mays* most likely consists of two

major phases: Initially, compatibility is established by general pathogenicity factors that suppress the PAMP-elicited basal plant defense responses during penetration (Doehlemann et al. 2008b). In a second phase of infection, fungal proliferation in the respective infected plant tissue is assured by specific subsets of effectors that are expressed in a strictly regulated spatial-temporal manner. Thus, the fungus is able to efficiently reprogram plant physiology and development of a specific organ tissue. It is tempting to speculate whether such a strategy is also deployed by other pathogenic organisms that infect plants as well as animal hosts.

9 Perspectives

The enormous progress made by the research community within the last years provided a substantial body of knowledge on the pathogenic development of fungal biotrophs and the regulatory mechanisms controlling these processes. A key role in establishment and maintenance of biotrophy is held by secreted effector proteins that directly interfere with plant targets and thereby control host defenses and metabolic reprogramming. In the *U. maydis*–maize pathosystem, the combination of genome sequencing, transcriptome profiling, and powerful reverse genetics identified a number of effectors with essential functions at distinct steps of pathogenic development. However, the molecular mechanisms of those effectors, their subcellular localization within the plant tissue as well as the cellular processes of the plant they target have not been clarified so far. For this reason, a major task for the near future will be to functionally analyze the *U. maydis* effectors that have important functions during the biotrophic interaction. This will require a smart combination of molecular, biochemical, and microscopic technologies together with bioinformatics resources. Comparative genomics of related smut fungi will be an important tool to identify conserved motifs in effectors that might give hints on their function as well as their localization. Effectome comparison in different smuts will also allow discriminating between host-specific proteins and factors being generally required for biotrophic interaction.

To study effector function in *U. maydis*, a major challenge will be to develop transient protein expression systems in maize that are compatible with the fungal infection. In other systems such as the *B. graminis*–barley interaction, both for transient overexpression and gene silencing, particle bombardment is an excellent tool to study the role of host genes on pathogen interaction (Eichmann et al. 2004). These assays also provide the possibility of a functional characterization of fungal effectors and their interactions with the respective plant targets. Since smut fungi only infect meristematic tissue, this kind of assay cannot be used to directly determine effects on fungal pathogenicity. The systemic VIGS system that recently has been described is a first step to allow a rapid functional characterization of putative effector-interacting maize proteins (van der Linde et al. 2010).

Candidate genes for effectors as well as for plant factors involved in the establishment and maintenance of compatibility often are selected on the basis of

transcript profiling approaches. However, all microarray data on the *U. maydis*–maize interaction has been gained from infected sections of whole plant organs, i.e., leaves and tassel. Particularly in the early stages of interaction only a low percentage of plant cells are actually in contact with fungal hyphae. In turn, the fungal material represents only a small fraction (<5%; Horst et al. 2010) in samples of infected leaves. This situation "dilutes" cell-specific effects and particularly subtle alterations in gene expression might be overlooked so far. Therefore, dissection of individual cells from different tissues will provide a much more detailed picture on the molecular processes associated with the intimate biotrophic interaction of *U. maydis* and its host.

Technical advances in confocal microscopy, -omics technologies, and the sequencing of reference genomes allowed completely new insights into biotrophic plant–pathogen interactions in the recent past. Now, high-throughput screening of effectors, comparative genome analysis, and in-depth transcriptome profiling based on second- and third-generation sequencing technologies will open new windows to unravel the hidden secrets of the friendliest of all antagonistic microbes – the biotrophs.

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Compatible Plant-Root Knot Nematode Interaction and Parallels with Symbiosis

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Abstract Among plant pathogens, the root-knot nematodes (RKN), *Meloidogyne* spp., are obligate biotrophic pathogens that can establish and maintain an intimate relationship with their host plants. They are able to induce the redifferentiation of root cells into hypertrophied and multinucleate feeding cells essential for their development. Hyperplasia of the surrounding feeding cells lead to the organogenesis of a typical root gall. In this chapter, we describe the complex interactions between RKN and their infected hosts. We highlight the progress in our understanding of host plant response during the compatible interaction focusing on key plant functions involved in giant cell ontogenesis. Throughout, parallels with symbiotic rhizobia–legume interactions are emphasized.

1 Introduction

Plants associate with a wide range of mutualistic and parasitic organisms, ranging from bacteria to nematodes. Obligate biotrophic plant pathogens develop stable, compatible associations with their hosts. A common feature in biotrophy is the development of specialized interfaces between the microorganisms and the plant cell over which nutrients are transferred (Harrison 1999). Therefore, it is quite conceivable that these interactions might have evolved certain common core components affecting cellular functions such as cell wall reorganization, membrane

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synthesis, metabolite fluxes or cytoskeleton rearrangements (Parniske 2000; Lipka and Panstruga 2005). It is a fact that the development and physiology of biotrophic interactions are specific and significantly different from each other (depending on the type of microorganism), but the ones occurring between plants and sedentary endoparasitic nematodes such as RKN, and the legume–*Rhizobium* symbioses are both leading to the formation of new root structures: the nodules on legume roots induced by symbiotic nitrogen-fixing bacteria and galls containing nematode feeding cells formed by RKN (Fig 1a). As in lateral root formation, both structures are initiated in the differentiated root zone and involve a reactivation of the cell cycle and a subsequent redifferentiation process (Grunewald et al. 2009b). Therefore, questions have been raised about molecular mechanisms and developmental pathways in the plant that ultimately determine the different outcomes of these interactions. Do these mechanisms share some common features or are they distinct phenomena? What are the processes that determine mutualism versus parasitism? This chapter reviews recent molecular insights into susceptible plant responses to RKN infection and comparisons between these two processes in legumes.

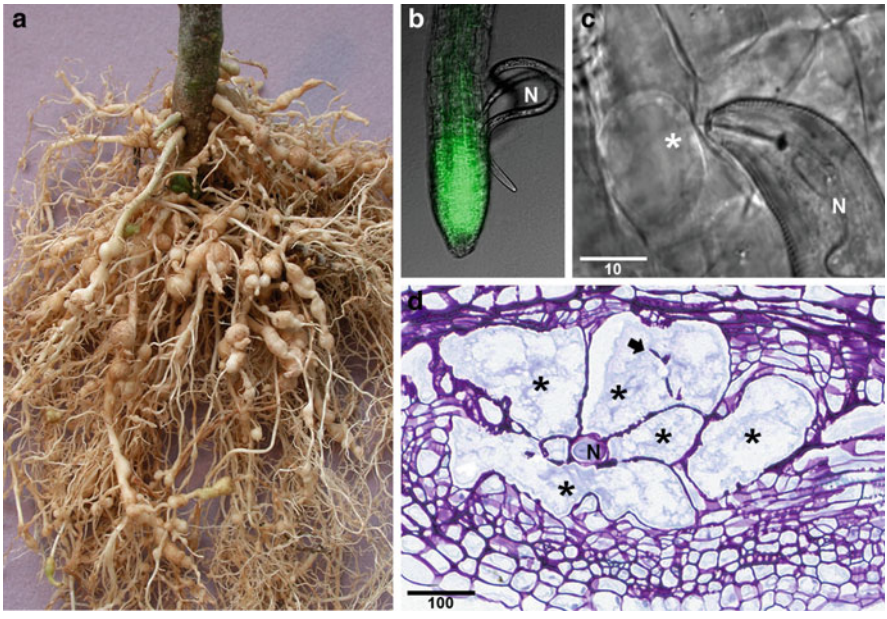

Fig. 1 Cellular responses to RKN infection. (**a**) Galls on tomato roots infected by *M. incognita*. (**b**) Infection of Arabidopsis root expressing MAP65-3:GFP by *M. incognita* J2 larvae. (**c**) Selection of root vascular cells and puncture by the J2 stylet. This cell will undergo a first nuclear division without cytokinesis leading to the formation of a binucleate cell. (**d**) Multinucleate and hypertrophied giant cells. Mini cell plates (*black arrow*) are visible. Sections through a gall at 15 days post-infection stained with toluidine blue. Asterisks, giant cell; N nematode; Bar = 10 μm (**c**) or 100 μm (**d**)

2 RKN Infection and Giant Cell Ontogenesis

In contrast to rhizobia which interact with legume species, the RKN host range encompasses nearly all flowering plants, leading to extensive crop loss (Abad and Williamson 2010). The wide host range (thousand of plant species) is associated with a worldwide distribution in all temperate and tropical areas making them extremely successful and damaging parasites (Trudgill and Blok 2001). RKN infect roots as microscopic vermiform second-stage juveniles (J2). Mobile J2s penetrate the root usually at the root elongation zone (Fig. 1b) and migrate between cells to reach the root apex and then enter into the plant vascular cylinder. Once in the stele, RKN select five to seven root parenchyma cells (Fig. 1c) and induce their redifferentiation into elaborate feeding cells, from which RKN puncture cytoplasmic contents with their protractible stylet (Fig. 1c). These multinucleate and hypertrophied feeding cells, named giant cells, result from synchronous nuclear divisions in the absence of cell division (Jones and Payne 1978). Recently, *in vivo* confocal microscopy revealed that failure to form functional cell plates following nuclear divisions may be attributed to a restricted outgrowth of the phragmoplast microtubule array that leads to the formation of particular mini cell plates (Fig. 1d) that do not extend across adjacent faces of the giant cell (Caillaud et al. 2008c). Giant cells continue to enlarge for 2–3 weeks, occupying the most part of the central cylinder. Mature giant cells may reach 100 times the size of a normal root vascular parenchyma cells and can contain up to 100 nuclei. Nuclei are highly amoeboid and show dispersed chromatin reflecting intensive gene transcription, as expected for an actively growing cell. Cytoplasmic content of giant cells increase greatly and normal vacuolization is lost. Wall ingrowths develop in contact with the xylem elements and an extensive wall labyrinth forms, increasing the exchange surface area at the associated membrane. These feeding cells function as specialized sinks supplying the nematode with nutrients throughout its life cycle. After 3–8 weeks, the pear-shaped female produces eggs that are released on the root surface. At the onset of giant cell induction, hyperplasia is observed where surrounding parenchyma vascular cells and pericycle cells divide to form the typical galls (Fig. 1a), which give RKN its name.

It is not yet understood how giant cells are induced, but it is certainly triggered by nematode secretions (Davis et al. 2004; Vanholme et al. 2004). Because RKN have such a large host range, they probably are able to hijack fundamental host functions. The recent characterization of a RKN secreted peptide able to interact with plant SCARECROW-like transcription factors gave new insights into the way nematodes can manipulate plant physiology to their own benefit (Huang et al. 2006). Full genome sequencing of two RKN provides new opportunities for studying plant–nematode interactions (Bird et al. 2009). Striking similarities between RKN and bacteria proteins have led to the discovery that some nematode parasitism proteins such as cell wall-degrading or -modifying enzymes, usually absent from animals, have been acquired by multiple independent lateral gene transfers from different bacterial sources (Scholl et al. 2003; Danchin et al. 2010).

3 Molecular Plant Response to RKN Infection

The routes by which nematodes manipulate their plant hosts to induce feeding cells are still not well understood. In the past few years, molecular and genetic approaches allowed substantial progress in our understanding of the host plant response during the compatible interaction. These approaches were based on expression analyses of candidate genes by promoter reporter fusions, in situ hybridization or RT-PCR, on promoter trap and on differential gene expression between healthy and infected root regions. The development of plant microarrays has made possible to generate large-scale patterns of plant gene expression during giant cell formation. Genome-wide expression profiling has been used to study the response of *Arabidopsis thaliana* or tomato to RKN infection (Bar-Orl et al. 2005; Hammes et al. 2005; Jammes et al. 2005; Fuller et al. 2007; Bhattarai et al. 2008). These studies identified a large number of genes being regulated in response to RKN infection, reflecting the complexity of nematode feeding site ontogenesis (Gheysen and Fenoll 2002; Bird and Kaloshian 2003; Caillaud et al. 2008b). More recently, material from giant cells was captured using laser microdissection at early stages following infection, showing that by 3 days post-inoculation (dpi) giant cells exhibit major gene regulation. This study confirmed the molecular distinctiveness of the giant cells within the gall and showed that most of the differentially regulated genes have no previously assigned function (Barcala et al. 2010).

These molecular approaches highlighted changes of some key plant processes such as cytoskeleton organization and cell cycle regulation (de Almeida Engler et al. 1999; de Almeida Engler et al. 2004; Favery et al. 2004; Caillaud et al. 2008c; Clement et al. 2009), extensive cell wall modification (Goellner et al. 2001; Vercauteren et al. 2002; Mitchum et al. 2004), hormone and defence responses (Lohar et al. 2004; Jammes et al. 2005; Karczmarek et al. 2008) and major reprogramming of plant metabolism (Wang et al. 2003; Hammes et al. 2006) (Table 1). Characterization of the genes specifically regulated during giant cell development is a first step towards understanding compatible plant–RKN interactions. Extensive analysis must be coupled with a detailed cellular expression pattern study, the characterization of knockout mutants and biochemical investigations, to more accurately dissect gene function during giant cell development. Up to date few mutants showing impaired nematode infection were characterized (Table 1). Recently, unique defects in giant cell ontogenesis were described in absence of regulators of microtubule (MT) or microfilament (MF) dynamics highlighting the importance of changes in the cytoskeleton architecture for a proper giant cell development (Caillaud et al. 2008c; Clement et al. 2009).

4 Cytoskeleton Organization

The plant cytoskeleton is a tremendously dynamic and flexible intracellular scaffold composed mainly of MTs and actin filaments (F-actin or MFs). It plays a central role in intracellular transports, cell division, cell differentiation and morphogenesis,

Table 1 Functional analysis of genes differentially expressed during RKN infection

Plant[a]	Gene name	Function	Expression		Phenotype[d]	References
			In gall[b]	In giant cells[c]		
Cell cycle						
At	*CDKA1/CDC2a*	Cell cycle regulator	UP	YES	RNAi pWRK23: less galls and egg masses	Niebel et al. (1996), de Almeida Engler et al. (1999) and Van de Cappelle et al. (2008)
At	*CDC2b*	Cell cycle regulator	UP	YES	–	de Almeida Engler et al. (1999)
At	*CYCA2;1*	Cell cycle regulator	UP	YES	–	de Almeida Engler et al. (1999)
At	*CYCB1;1*	Cell cycle regulator	UP	YES	–	Niebel et al. (1996) and de Almeida Engler et al. (1999)
At	*PRL*	DNA replication initiation	UP	YES	–	Huang et al. (2003)
Mt	*CCS52a*	Endoreduplication	UP	YES	–	Favery et al. (2002) and Koltai et al. (2001)
Cytoskeleton						
At	*AtFH6*	Formin	UP	YES	KO: no effect	Favery et al. (2004)
At	*AtFH10*	Formin	UP	YES	KO: no effect	Jammes et al. (2005)
At	*ADF2*	Actin depolymerizing factor	UP	YES	inducible RNAi: aborted giant cells	Clement et al. (2009)
At	*ADF3*	Actin depolymerizing factor	UP	–	–	Fuller et al. (2007)
At	*ADF4*	Actin depolymerizing factor	UP	NO	–	Clement et al. (2009)
At	*ACT2*	Actin	UP	YES	–	de Almeida Engler et al. (2004)
At	*ACT7*	Actin	UP	YES	–	de Almeida Engler et al. (2004)
At	*MAP65-3*	Microtubule Associated Protein	UP	YES	KO: aborted giant cells	Caillaud et al. (2008c)
At	*TUB1*	β-tubulin	UP	–	–	Lilley et al. (2004)
At	*Tubulin β, α*	Tubul ns	UP	YES	–	de Almeida Engler et al. (2004)

(continued)

Table 1 (continued)

Plant[a]	Gene name	Function	Expression		Phenotype[d]	References
			In gall[b]	In giant cells[c]		
Cell wall						
At	*CEL1*	Cellulase	UP	YES	–	Mitchum et al. (2004)
At	*PAE*	Pectin acetylesterase	UP	YES	–	Vercauteren et al. (2002)
Np	*Extensin*	Cell wall protein	UP	YES	–	Niebel et al. (1993)
Nt	*NtCEL7, 8*	1-4 endoglucanase	UP	YES	–	Goellner et al. (2001)
Nt	*NtCEL7*	1-4 endoglucanase	UP	YES	–	Wang et al. (2007)
Nt	*NtCEL2*	1-4 endoglucanase	UP	YES	-	Goellner et al. (2001)
Sl	*EXPA5*	Expansin	early	NO	AS lines: less egg masses	Gal et al. (2006)
Hormone response						
Sl	*DIAGEOTROPICA*	Auxin response	–	–	KO: more resistant	Richardson and Price (1984)
At	*ARR5*	Cytokinin response	UP	NO	–	Lohar et al. (2004)
At/Zm	*AtCKX3/ZmCKX1*	Cytokinin oxidase	–		OE: 50% less galls	Lohar et al. (2004)
Mt	*ERF1.1*	Ethylene response factor	–	–	OE: no effect	Anderson et al. (2010)
Lj	*ETR1*	Ethylene resistant	–	–	Transgenics carrying the Arabidopsis dominant etr1.1: No effect	Lohar and Bird (2003)
Gm	*GH3/DR5*	Auxin responsive	UP	YES	–	Hutangura et al. (1999) and Karczmarek et al. (2004)
At	*AUX1*	Auxin transport	UP	–	–	Mazarei et al. (2003)
Metabolism						
Gh	*LEA14*	Late embryogenesis abundant protein	UP	NO	–	Gheysen et al. (1996)
At	*RHA1/ATRAB5A*	Small GTP binding protein	UP	NO	–	Vercauteren et al. (1998)
At	*GRP7*	Oleosin	UP	–	–	Karimi et al. (2002)

At	*ATAO1*	Diamine oxidase	UP	–	–	Møller et al. (1998)
Pa	*HEMOGLOBIN*	Oxygen transport	UP	–	–	Ehsanpour and Jones (1996)
At	*mUCP*	Mitochondrial uncoupling protein	UP	YES	–	Vercauteren et al. (2001)
Sl	*LEMMI9*	Late embryogenesis abundant protein	UP	YES	–	Escobar et al. (1999)
Ha	*HSP17.7G4*	Heat shock protein	UP	YES	–	Escobar et al. (2003) and Barcala et al. (2008)
Ha	*HSP17.6G2*	Heat shock protein	UP	YES	–	Barcala et al. (2008)
At	*RPL16A*	Ribosomal protein	UP	–	–	Lilley et al. (2004)
At	*PAG1*	20S proteasome subunit	UP	YES	–	Vercauteren et al. (2001)
At	*RPE*	Ribulose phosphate epimerase	UP	YES	KO: no galls	Favery et al. (1998)
At	*PGM*	Phosphoglycerate mutase	UP	–	–	Mazarei et al. (2003)
Sl	*HMG2*	Hydroxy-methylglutaryl CoA reductase	UP	YES	–	Cramer et al. (1993)
At		Endomembrane protein	UP	YES	–	Vercauteren et al. (2001)
At	*DapDC*	Diaminopimelate decarboxylase	UP	YES	–	Vercauteren et al. (2001)
Wound response and defense						
At	*PAL1*	Phenylalanine ammonia-lyase	DOWN	NO	–	Goddijn et al. (1993)
St	*GST1*	Glutathione-S-transferase	UP	–	–	Strittmatter et al. (1996)
St	*WUN1*	Wound response	UP	–	–	Hansen et al. (1996)
Sl	*TSW12*	Lipid Transfer Protein	UP	YES	–	Gheysen and Fenoll (2002)
At	*POX*	Peroxidase	UP	YES	–	Vercauteren et al. (2001)
At	*TPI*	Trypsin protease inhibitor	UP	YES	–	Vercauteren et al. (2001)

(continued)

Table 1 (continued)

Plant[a]	Gene name	Function	Expression		Phenotype[d]	References
			In gall[b]	In giant cells[c]		
At	*TPI*	Trypsin protease inhibitor	DOWN	NO	KO: no effect	Jammes et al. (2005)
At	*COMT1*	Caffeate O-methyltransferase	UP	YES	KO: no effect	Quentin et al. (2009)
Nt	*OMT*	O-methyl transferase	–	–	AS lines: less egg masses	Wuyts et al. (2006)
At	*LTP*	Lipid Transfer Protein	UP	–	–	Fuller et al. (2007)
At	*PR1*	Pathogenesis-related protein	–	–	OE: less galls	Hamamouch et al. (2010)
At	*PR2*	β-1,3-glucanase	–	–	OE: no effect	Hamamouch et al. (2010)
At	*PR3*	Endo-chitinase	–	–	OE: no effect	Hamamouch et al. (2010)
At	*PR4*	Endo-chitinase	–	–	OE: no effect	Hamamouch et al. (2010)
At	*PR5*	Thaumatin-like	–	–	OE: no effect	Hamamouch et al. (2010)
Tr	*CHS1–HS3*	Chalcone synthase	UP	YES	–	Hutangura et al. (1999)
At	*DFG (tt3)*	Dihydroflavono reductase	–	–	KO: no effect	Wuyts et al. (2006)
At	*CHS (tt4)*	Chalcone synthase	–	–	KO: no effect	Wuyts et al. (2006)
Mt	*CHS*	Chalcone synthase	–	–	RNAi: reduced gall size and cell number per gall	Wasson et al. (2009)
At	*CHI (tt5)*	Chalcone isomerase	–	–	KO: no effect	Wuyts et al. (2006)
At	*F3′H (tt7)*	Flavonoid 3′ hydroxylase	–	–	KO: no effect	Wuyts et al. (2006)
At	*pC4H-F5H*	Ferulic acid 5-hydroxylase	–	–	OE: less females	Wuyts et al. (2006)
Zm	*LOX*	Lipoxygenase	–	–	KO: egg number increase	Gao et al. (2008)
Nt	*PAP2*	MYB transcription factor	–	–	OE: gall and egg number increase	Wuyts et al. (2006)

Nt	*PAL*	Phenylalanine ammonia-lyase	–	–	OE: no effect	Wuyts et al. (2006)
Transport						
At	*AAP6*	Amino acid transporter	UP	YES	–	Hammes et al. (2005)
At	*CAT6*	Amino acid transporter	UP	YES	KO: no effect	Hammes et al. (2006)
At	*AMT1;2*	Ammonium transporter	DOWN	NO	–	Fuller et al. (2007)
At	*α-TIP1-1*	Tonoplast aquaporin	DOWN	NO	–	Goddijn et al. (1993)
At	*PIP2.5*	Plasma membrane aquaporin	UP	YES	–	Hammes et al. (2005)
At	*ARSK1*	Serine/threonine kinase	UP	–	–	Lilley et al. (2004)
Nt	*TobRB7*	Tonoplast aquaporin	UP	YES	–	Opperman et al. (1994)
Miscellaneous						
Mt	*ENOD40*	Repetitive proline-rich proteins early nodulin	UP	NO	OE: gall number increase	Favery et al. (2002)
Mt	*ENOD11*	Early nodulin	UP	NO	–	Boisson-Dernier et al. (2005)
At	*LBD41*	Lateral organ boundary domain	UP	–	–	Fuller et al. (2007)
Mt	*KNOX*	Transcription factor	UP	YES	–	Koltai et al. (2001)
Mt	*PHAN*	Transcription factor	UP	YES	–	Koltai et al. (2001)
Lj	*HAR1*	Clavata-like LRR-RLKinase	–	–	KO: gall number increase	Lohar and Bird (2003)
At	*SRS5*	Short internode (SHI)-related	UP	YES	–	Barcala et al. (2010)
At	*WRKY23 (Att0001)*	WRKY transcription factor	UP	YES	KO: no effect	Barthels et al. (1997) and Grunewald et al. (2008)

Are indicated genes for which data on expression were obtained/confirmed at the cellular level using GUS- or GFP-reporter gene or RNA in situ hybridization or for which knock-out, -down or overexpression were studied. [a]*At Arabidopsis thaliana*; *Gh Gossypium hirsutum*; *Gm Glycine max*; *Lj Lotus japonicus*; *Mt Medicago truncatula*; *Np Nicotiana plumbaginifolia*; *Nt Nicotiana tabacum*; *Pa Parasponia andersonii*; *St Solanum tuberosum*; *Sl Solanum lycopersicum*; *Tr Trifolium repens*; *Zm Zea mays*. [b]UP means upregulated; DOWN downregulated gene expression. [c]Grey shade indicate data on the subcellular localization of the protein in giant cells. [d]Knock-out (KO), anti-sense expression (AS) or overexpression (OE) effects are indicated; –, not determined

and is essential in regulating establishment of plant–pathogen interactions and symbiosis. Immunostaining and use of fluorescent markers for observation of the cytoskeleton structures within the giant cells revealed major rearrangements of both MFs and MTs during nematode feeding sites formation (de Almeida Engler et al. 2004; Caillaud et al. 2008a; de Almeida Engler et al. 2010). Chemical blocking of the actin or MT cytoskeleton dynamics (stabilization or breakdown) during feeding cell initiation resulted in the arrest of proper giant cell development and consequently nematode development (Wiggers et al. 2002; de Almeida Engler et al. 2004). Microtubule-associated proteins (MAPs) and acting-binding proteins (ABPs) play essential roles in controlling cytoskeleton dynamics and organization. Proteins implicated in giant cell actin and MT cytoskeleton reorganization were identified. Formins and actin-depolymerising factors (ADFs) are major regulator of actin dynamics and architecture. Three genes coding for Arabidopsis formins AtFH6, AtFH1 and AtFH10 were upregulated in galls upon *M. incognita* infection, and AtFH6 was shown to be anchored and uniformly distributed throughout the giant cell plasma membrane (Favery et al. 2004). It is hypothesized that formins regulate assembly of actin cables guiding the vesicle trafficking needed for extensive plasma membrane and cell wall biogenesis and therefore giant cells isotropic growth. Recently, ADF2 has been shown to be instrumental to the successful RKN infection. Knocking down *AtADF2* resulted in a decrease in F-actin turnover responsible for the stabilization and bundling of the MFs. This actin cytoskeleton stabilization did not allow nematodes to mature into females (Clement et al. 2009). A unique defect in giant cell formation in a *map65* Arabidopsis mutant was described (Caillaud et al. 2008c). Detailed functional analyses of MAP65-3 showed that it plays a key role in the organization of MT arrays during both mitosis (spindle morphogenesis) and cytokinesis (phragmoplast expansion) and was associated with mini cell plates (Fig. 1d) formed between daughter nuclei during cytokinesis initiation in developing giant cells. In the absence of MAP65-3, giant cells started to develop but accumulation of mitosis defects (cell wall stubs and connected nuclei) during repeated nuclear division prevented the development of functional feeding cells. Giant cells did not complete their differentiation process and were eventually destroyed impairing the maturation of the infecting nematodes.

While cytoskeleton reassembly during symbiotic interactions has been studied extensively (Takemoto and Hardham 2004; Schmidt and Panstruga 2007), identification of components regulating this process is at its beginning. The two proteins NAP1 and PIR1 of the SCAR/WAVE complex responsible for the activation of the ARP2/3 complex involved in actin nucleation and polymerization are the only regulators that have been identified as being directly involved in the invasion of *Lotus japonicus* roots by *Mesorhizobium loti* (Yokota et al. 2009).

5 Cell Cycle Regulation and Endoreduplication

The first sign characteristic of giant cell induction is the cell cycle (re)activation in parenchyma vascular cells. Giant cells will then be subjected to synchronous repeated mitosis without complete cytokinesis. Nodule formation is initiated by

dedifferentiation of root cortical cells followed by cell proliferation, establishing a cluster of meristematic cells that give rise to the nodule primordium (Patriarca et al. 2004; Crespi and Frugier 2008). To initiate organogenesis, both nematodes and symbiotic bacteria recruit cell cycle regulators, such as cyclins or cyclin-dependent kinases, at early stages of the interactions (Table 1) (de Almeida Engler et al. 1999; Barcala et al. 2010; Maunoury et al. 2010). In nodule, following division, cells undergo endoreduplication – repeated cycles of DNA replication without mitosis – allowing their differentiation into enlarged symbiotic cells (Vinardell et al. 2003). Recently, Maunoury et al. (2010) hypothesized that it is endoreduplication that may act as a transcriptome switch for the cell to evolve from progenitor primordium or meristematic cell to a symbiotic cell. High expression of CCS52 was observed in endoreduplicating nodule tissues of *M. truncatula* (Cebolla et al. 1999). CCS52 promotes endoreduplication by activation of the anaphase-promoting complex (APC) resulting in mitotic cyclin destruction and mitosis arrest. Upregulation of CCS52 was also observed in giant cells and their surrounding cells (Koltai et al. 2001; Favery et al. 2002) suggesting that endoreduplication process occurs in giant cells. Its function still remains unclear but should represent an important process in the development of functional giant cells to support the enhanced metabolic demands imposed by RKN. More generally host endoreduplication is an emerging theme in biotroph–plant interactions (Wildermuth 2010).

6 Plant Hormone Response

Since plant organogenesis is largely regulated by plant hormones, researches have focused on whether microorganisms directly or indirectly manipulate the plant hormone balance to induce root nodule or gall formation. Using an auxin-responsive promoter GH3 (or its synthetic derivative DR5) fused to the reporter gene GUS, an early and transient increase in auxin response was detected in giant cells and in white clover nodule primordia (Mathesius et al. 1998; Hutangura et al. 1999). Nodule initiation in indeterminate legumes was preceded by an inhibition of acropetal auxin transport. Similarities in the proteomes of root cells during the early step of nodulation and after auxin application suggest that auxin mediates substantial protein changes in nodulation initiation (van Noorden et al. 2007). Interestingly, the *M. truncatula* supernumerary nodules mutant *sunn* presents an altered auxin level that correlates with nodules number and protein accumulation. During RKN infection, auxin accumulation was suggested to trigger gall formation and was associated with an induction of the flavonoid pathway as observed in rhizobia nodules. Several flavonoids are known to be putative auxin transport inhibitors. However, if flavonoid-deficient roots did not form nodules they showed no difference in the number of galls. Galls induced on flavonoid-deficient roots formed normal giant cells, but were shorter and characterized by reduced numbers of dividing surrounding cells (Wasson et al. 2009).

The increased local auxin response in neoformed organs can result from increased auxin influx and/or an inhibition of auxin efflux. The induction observed in microarray studies (Hammes et al. 2005; Jammes et al. 2005) of two Arabidopsis genes coding for the auxin influx carriers AUX1 and LAX3 is consistent with a role for auxin in the establishment of RKN. During nodule initiation a functional auxin transport system is required since silencing of several *PIN* genes result in a reduced nodulation in *M. truncatula* (Huo et al. 2006). How auxin importers and exporters are regulated during nodulation and gall formation is still unknown (Grunewald et al. 2009b). The first study on the role of the auxin transport during plant–parasitic nematode interaction was done on cyst nematodes, another group of sedentary parasitic nematode. Manipulation of both the expression and the subcellular polar localization of PIN auxin transporters by cyst nematodes facilitates the initiation and development of their multinucleate feeding sites, named syncytia (Grunewald et al. 2009a). RKN giant cells differ significantly from syncytia that result from cell wall dissolution and integration of surrounding cells. Moreover, in contrast to cyst nematodes which induce significantly less infection on several auxin signalling mutants (Goverse et al. 2000; Grunewald et al. 2009a), only one hormone mutant, the tomato *diageotropica*, has been reported to alter RKN parasitism (Richardson and Price 1984). As auxin is an important player in plant organogenesis it is likely that auxin and auxin transport are targeted by RKN.

Not only changes in the host auxin would be required for nodules and giant cells/gall induction but also cytokinins have emerged as an important player in these two interactions. Using the Arabidopsis cytokinin-responsive *ARR5* gene as a marker, a role for cytokinin has been highlighted during the first steps of nodule initiation and gall formation. This gene is, however, repressed in giant cells and in mature gall and determinate nodules. Thus, cytokinins can play a role either as stimulator or inhibitor of cell division. In addition, decreasing cytokinin levels by expressing a cytokinin oxidase (*CKX*) resulted in a reduction of both nodules and gall numbers on transgenic hairy roots (Lohar et al. 2004). A strict correlation between an elevated cytokinin level and *KNOX* gene expression has been observed, suggesting that cytokinins may either regulate *KNOX* expression or be regulated by KNOX (Bird and Kaloshian 2003). KNOX and the Myb transcription regulator PHAN are required for normal meristem maintenance and induced in both *M. truncatula* galls and nodules again illustrating similarities between these two organs.

Phytohormones contribute also to modulate defence process. The role of the signalling molecules jasmonic acid (JA) and salicylic acid and ethylene is well described in shoots, however much less is known about their role in roots. Recent advances on symbiosis and nematode interactions were recently reviewed (Gutjahr and Paszkowski 2009). Thus, tomato susceptibility to RKN would require an intact JA signalling pathway (Bhattarai et al. 2008).

7 Defence-Related Signalling Compounds

During invasion RKN cause little damage to the root as they migrate between the cells. Still, a rapid and transient production of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), is detected at the time of

nematode invasion. No significant oxidative burst was detectable at the time of giant cell induction and at later time points (Melillo et al. 2006; Das et al. 2008). ROS are also produced in host plants during interaction with rhizobia notably in the infection process (Santos et al. 2001). So the bacteria have to protect themselves from these defence molecules and bacterial strains with impaired capacity to detoxify H_2O_2 have deficiencies in their symbiotic capacities. Despite negative effects, ROS has been reported as signalling molecules in the control of nodulation process, and a threshold level of ROS would be required for a harmonious nodule development (Pauly et al. 2006). Additional major molecules involved in the regulation of the cellular redox state, nitrogen monoxide (NO) and glutathione (GSH) are detected in response to rhizobia and required in proper nodule development (Frendo et al. 2005; del Giudice et al. 2011). Unfortunately, the role of GSH and NO in giant cell formation has not yet been investigated.

Microarray analysis showed that the successful establishment of RKN is associated with the suppression of plant defence responses in *A. thaliana* (Jammes et al. 2005). Most of the defence-related genes such as *WRKY* transcripts and genes associated to the JA/ethylene-dependent pathways (*EIN3*, *ERF1*, *PR4*) were repressed particularly at later stage of gall formation. Expression of maize lipoxygenase *LOX3* and Arabidopsis pathogenesis-related genes *PR-1*, *PR-2*, *PR3* and *PR5* peaked at 7 or 9 dpi, respectively, in RKN infected roots (Gao et al. 2008; Hamamouch et al. 2010). The observed downregulation of defence genes from host plants suggests an active modulation of the plant response by RKN as reported in different plant–pathogen interactions (Abramovitch and Martin 2004). Defence suppression also appears to play an important role in symbiotic plant–microbe interactions. The NopL effector of *Rhizobium* sp. NGR234 suppresses *PR* gene expression when expressed in tobacco or *L. japonicus* (Bartsev et al. 2004).

8 Additional Nodulins Involved in RKN Parasitism

Strikingly, certain early nodulin genes (*ENOD*) have been shown to respond to RKN infections. ENOD40 is also stimulated in galls from *M. truncatula* induced by RKN but not in giant cells (Favery et al. 2002). ENOD40 is involved in both the initiation and the stimulation of cortical cell division for nodule formation. It may play a similar role in stimulating the proliferation of cells around giant cells for gall formation. In the same way, *ENOD11*, a cell wall proline-rich nodulin gene early expressed during nodulation is stimulated during RKN infection (Boisson-Dernier et al. 2005). *ENOD11* expression was detected using GUS activity exclusively in cells surrounding the developing giant cells, as it was observed for *ENOD40*. The expression patterns of these two nodulins suggest a role in cell-to-cell communication events between giant cells and vascular surrounding tissues. A larger scale comparison using macroarrays revealed that out of 192 nodule-expressed genes only two genes, nodulin 26 and cyclin D3, were found to be upregulated upon RKN infection (Favery et al. 2002).

Intriguingly, the host perception machinery involved in bacterial nodulation process would be also involved in RKN infection. By comparing responses of mutants of Nod factor receptors *nfr1*, *nfr5*, and *symRK* and wild-type plants in *L. japonicus*, the ability of RKN to establish feeding sites and reproduce has been shown to be markedly reduced in the mutant lines (Weerasinghe et al. 2005). In addition, root-hair waviness and branching were detected in *L. japonicus* RKN-infected roots suggesting possible common signal transduction pathway targeted by infective RKN J2 and Nod factors in developing root hairs.

9 Conclusions

There are evidences for partial overlaps in plant microbe signalling at the cellular and molecular levels. However, comparison between galls and nodules should take into account that both organs are complex and contain specialized zones or cells. Transcriptomics approach combined with laser capture microdissection will greatly refine and expand analyses of giant cells and their surrounding cells and nodule specific zones. A first comparison between giant cells and the infection zone II of *M. truncatula* nodules indicated an overlap of 10% and 35% of genes commonly upregulated or downregulated, respectively (Damiani et al. in preparation). These first data highlight the recruitment of common plant pathways for infection by pathogen and symbiont as well as the involvement of processes specific for each type of interactions. Development of integrative 'omics' approach including proteomics and metabolomics will reveal the concurrent similarities and differences in plant signalling pathways leading to different organs and developmental outcomes as nodules or roots galls. Furthermore, substantial progress will also be made in the next years in characterizing the direct host targets of secreted RKN effectors and studying whether small RNAs function as key player providing unique insights into manipulated plant cellular processes during giant cell ontogenesis. Finally, determining how a nematode selects particular root cells and modifies them to serve as a feeding cell will enhance our understanding of plant cell development and will contribute to offer alternative to nematicides and novel approaches requested to protect plant against plant parasitic nematodes.

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