1 **Reconsidering Mutualistic Plant-Fungal Interactions through the Lens of**
2 **Effector Biology**

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26 *Abstract*

27 Mutualistic plant-fungal interactions have shaped the evolution of plant life on land.
28 In these intimate associations, fungal hyphae grow invasively within plant tissues.
29 Despite this invasion, these fungi are not repulsed leading to a great deal of research
30 focused on the signals exchanged between mutualistic fungi and their host plants in an
31 effort to understand how these relationships are established. In this review, we focus
32 on one type of signal used by mutualistic fungi during symbiosis: effector proteins.
33 These small secreted proteins have recently been found to be used by a broad range of
34 beneficial fungi to alter the physiological status of the plant host such that symbiosis
35 is favoured. We discuss how the role of these novel proteins has altered our vision of
36 how the “mutualistic” lifestyle evolved in fungi: rather than being perceived as
37 beneficial by their plant hosts, these microbes currently viewed as “beneficial” may
38 actually be overcoming the defences of their plant hosts in a mechanism originally
39 thought to be unique to pathogenic microbes.

40

41 *Introduction*

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43 As we deepen our understanding of how ecosystems sustainably function, the one
44 pattern that continues to re-emerge is the interdependence of all living things upon
45 each other. Mutualistic interactions between two distinct organisms, such as between
46 plants and mycorrhizal fungi, would seem to be the epitome of a beneficial inter-
47 dependence where two species have co-evolved to benefit themselves and their
48 partner....or is it?

49

50 The very existence of terrestrial plant life as we know it, has been attributed to the
51 mutualistic interaction between early plants and fungi [1]. From their birthplace in
52 oceanic and inter-tidal regions, the first land plants had to cope with a high carbon
53 dioxide atmosphere, nutrient poor soils and severe fluctuations in both temperature
54 and water availability [2, 3]. The original postulate was that the fungus that enabled
55 diversification of plants onto land was a “mycorrhizal” oomycete [1]. As fossil
56 evidence continued to emerge, it was proposed that rather than an oomycete, early
57 land plants were instead colonised by arbuscular mycorrhizal (AM) fungi [4]. The
58 AM symbiosis is now considered ancestral in land plants [5], and Strullu-Derrien *et*
59 *al.* [6] provided further support for this view, based on new fossils from the 407 Myrs
60 old Rhynie Chert. Recently, it has been shown that extant basal land plants, such as
61 liverworts, hornworts and lycopods, associate with Mucoromycetes [7] in a symbiosis
62 whose mutualistic nature is suspected [8]. No matter the exact identity of the fungus
63 that was associated with these early plants, it is indisputable that the first land plants
64 were heavily colonised by fungi. How this very intimate association evolved, and
65 why it was not repulsed by the plant, is still not fully comprehended even in modern-
66 day mutualistic fungal associations [9**]. Philosophically, at the base of all plant-

67 fungal mutualistic associations, is the assumption that there is a mutually beneficial
68 give-and-take in these relationships: the fungus provides nutrients such as nitrogen
69 and phosphorus to the plant in return for photosynthetically fixed carbon. There is
70 some evidence for this in AM fungal symbioses [10-12] with the plant diverting more
71 carbon to fungal symbionts that provide the most growth limiting nutrients. It is
72 unknown, however, if similar relationships exist in other mycorrhizal associations.
73 The exchange of nutrients is also a fairly late-stage occurrence in these interactions.
74 Preceding this is an initial exchange of signals between the fungus and the plant
75 followed by the initial physical contact between the fungus and plant roots. In
76 mycorrhizal plant-fungal associations, the molecular dialogue that passes between the
77 two organisms is necessary to fungal invasion of plant tissues [13-17]. We will focus
78 in this review on our most recent advances in understanding these diffusible signals
79 during the early stages of the interaction between plants and beneficial fungi.
80 Specifically, we will reconsider the role of fungal proteins named ‘effectors’ in
81 dialoguing with the plant during symbiosis and reflect upon how the role of these
82 curious proteins has fundamentally changed our concept of how the mutualistic
83 lifestyle arose in fungi.

84 85 *Effective Host Take-Over*

86 Hundreds of species of endophytic, mutualistic and pathogenic Fungi associate with
87 plant tissues. Pathogenic fungi have evolved the ability to manipulate the host plant
88 metabolism such that the fungus receives all of its nutrient requirements without
89 benefiting the plant host. It has long been known that pathogenic microorganisms
90 have been able to succeed in such a manner due to the evolution and deployment of
91 small secreted proteins (SSPs) of unknown function, so-called “effector” proteins

[18]. These SSPs are produced by the pathogen during plant colonisation and they alter plant functioning in favour of fungal growth in plant tissues. Interestingly, however, the sequencing of the first mycorrhizal fungal genome (*Laccaria bicolor*, 19*) demonstrated that beneficial fungi also encoded, and expressed, effector-like SSPs during the interaction under pot- and microcosm-based colonisation conditions. Evidence for the expression of effector-like mycorrhiza-induced small secreted proteins (MiSSPs) has also been found *in situ* in wild-harvested *Piloderma-Pinus* ectomycorrhizal root tips [20**].

As the number of genomes sequenced and annotated for beneficial fungi has grown, so too has the evidence for the evolution of effector-like proteins that facilitate plant colonisation in a large number of mycorrhizal fungal lineages [9**]. An analysis of the genes differentially expressed during mutualistic plant-fungal interactions spanning ecto-, orchid and ericoid mycorrhizal fungi found that 1-12% of the SSP genes encoded in these genomes were highly induced during a symbiotic interaction (Figure 1A). Interestingly, the brown-rot fungus *Serpula lacrymans*, which is phylogenetically-related to ectomycorrhizal fungi within the Boletales [21], has a similar complement of SSP sequences encoded in its genome when compared to mycorrhizal fungi (Figure 1B) but it does not display the same level of SSP transcriptional induction when grown on wood or root tissues (Figure 1A; 9**). Therefore, the high percentage of effector-like SSPs induced during mycorrhization, and the lack of similar gene expression profiles in saprotrophic fungi, suggests that a large part of these proteins are necessary for mutualistic interactions just as they are for pathogenic plant-fungal interactions. Although several of these MiSSPs may play

a structural role during the formation of the symbiosis interfacial matrix, it is believed that many are effector-like proteins.

Proof-of-Concept: Effector-like proteins alter plant host function during beneficial plant:microbe interactions

Since their identification, intensive efforts have been underway to characterise the function of effectors encoded by mutualistic fungi. To date, the function of a handful have been published. These effectors, however, span a range of beneficial microbes from the AM fungus *Rhizophagus irregularis* (SP7; 22**), through to *Piriformospora indica* (PIIN29; 23) to the ectomycorrhizal fungus *Laccaria bicolor* (MiSSP7; 24**, 25**). We will detail the current state of knowledge concerning these effectors here.

During the colonisation of host plants, AM fungal hyphae grow inter-cellularly running along the length of the root. At intervals, these hyphae will penetrate the cell wall and begin to develop an arbuscule: a mass of finely branched hyphae surrounded by a peri-arbuscular membrane. The presence of the membrane ensures that the fungus never penetrates into the cytoplasm of the cell and it is across the surface area of this membrane that the plant and the fungus exchange nutrients. During the early stages of the interaction with *Medicago truncatula*, *R. irregularis* expresses the effector-like protein SP7 [22**]. This protein was found to be secreted from the fungal hyphae, crosses the peri-arbuscular membrane and enters the plant cell. Yeast-two hybrid assays uncovered that SP7 interacted with the host protein ERF19, a key transcription factor in the ethylene signalling pathway. Ethylene has been implicated in plant defence during symbiosis [26, 27*, 28] thus the repression of the ethylene pathway by SP7 may lower plant defences to allow the interaction. Supporting this,

ERF19 was found to be highly up-regulated during pathogenic plant-microbe interactions but only transiently expressed during the interaction between *R. irregularis* and its plant host. [22**]. Therefore, SP7 is a key effector protein of *R. irregularis* that alters plant hormone signalling to promote fungal growth within plant tissues to establish and maintain biotrophy.

P. indica is a beneficial fungal endophyte that grows extensively within the roots of its host plants and increases plant nutrient uptake [29], biomass accumulation and stress tolerance [30, 31]. *P. indica* exhibits a bi-phasic colonisation strategy, initially colonising live host tissues in a similar manner to AM fungi and later in dead or dying host cells [32, 33**, 34, 35]. Like other microbes that interact with plant hosts, *P. indica* encodes a large number of effector-like SSPs [33**]. One of these, PIIN29, which displays sequence homology to E3 ligases, was recently described as being induced during the interaction [23]. Over-expression of this protein in plant cells led to an increase in colonisation of *P. indica* in the roots of *Arabidopsis thaliana*, likely due to its ability to suppress the expression of resistance genes, the generation of reactive oxygen species and deposition of callose. Therefore, the effectors of *P. indica* appear to divert host cellular defense pathways, although the exact mechanism has yet to be elucidated.

In forest environments, ectomycorrhizal (ECM) fungi are one of the predominant mutualistic partners of trees and a number of shrubs. As opposed to the previous two fungi discussed, ECM fungi never enter plant cells but rather grow on the surface of roots and exchange nutrients in the intra-cellular space. Within this classification of fungi, *L. bicolor* was the first to have its genome sequenced [19*] and was the first to

have one of its symbiotic effectors functionally characterised [24**, 25**]. This effector, MiSSP7, is one of the most highly up-regulated genes in *L. bicolor* during the interaction with a plant host [19*, 36], is secreted from the hyphae of *L. bicolor*, and is taken up into the plant cell via endocytosis. After entry into the *Populus* cells, the majority of MiSSP7 is found localised in nuclei where it interacts with PtJAZ5 and PtJAZ6, JASMONATE-ZIM DOMAIN (JAZ) repressor proteins playing a central role in the jasmonate signaling cascade [25**]. This physical interaction protects these JAZ proteins from degradation by the 26S proteasome and leads to the repression of transcription of a group of genes normally induced when JA is accumulating. The stabilisation of these JAZ proteins is necessary for the establishment of symbiosis between *L. bicolor* and *P. trichocarpa* as a reduction in PtJAZ6 inhibits fungal ingrowth into the root apoplast of *P. trichocarpa* and as over-expression of *PtJAZ6* can complement the loss of MiSSP7 production in *L. bicolor* [25**]. Therefore, like in *R. irregularis*, *L. bicolor* produces an effector protein that alter hormone signalling in the host plant to promote symbiosis. Interestingly, pathogenic bacteria and oomycetes also produce effector proteins that target JAZ proteins during disease formation [37*, 38, 39*] suggesting that hormonal regulation is a central signalling ‘hub’ that is a key step in plant function that determines the fate of plant-microbe interactions.

Effector sequence diversity may mask large-scale functional redundancy

The presence of so many MiSSPs across most mutualistic fungal genomes sequenced to date begs the question of their evolutionary origin. Unfortunately, like their pathogenic counterparts, the answer to this remains shrouded in mystery as many of the newly identified MiSSP genes appear to be restricted to a single mycorrhizal

species [9**]. Even in a comparison of closely related *Laccaria* species (*L. bicolor* vs. *L. amethystina*; last common ancestor 20 Mya), only ~30% of MiSSP sequences are conserved.

Such weak conservation of MiSSP gene sequences would suggest that these proteins have evolved very specific roles in establishing and negotiating symbiosis with specific plant partners. This conclusion, however, may not be fully justified. As described above, the effector MiSSP7 of *L. bicolor* targets a similar host protein set as the effectors of very different pathogenic microbes [37*, 38, 39*]. Also, when comparing the expression of MiSSP genes of *L. bicolor* in mutualistic symbiosis with *Populus trichocarpa* (an angiosperm) and with *Pseudotsuga menziesii* (a gymnosperm), a nearly identical panel of MiSSP genes are induced despite the radically different hosts [36]. These results would suggest that effectors and other symbiosis-related SSPs within mycorrhizal genomes, rather than being produced via diversifying evolutionary processes, are the result of convergent evolution whereby phylogenetically unrelated genes encode proteins that have the same function derived through conservation of specific protein folding or active domains. A similar situation has already been found in the pathogenic bacteria *Pseudomonas syringae* and the oomycete *Hyaloperonospora arabidopsidis* which encode evolutionarily distant effector proteins that target and interact with the same plant protein [37*].

Therefore, while at the genomic level the effector repertoire of mutualistic organisms may appear to be distinct, the large majority of these effector and other symbiosis-related SSPs may serve the same functional process during symbiosis.

Despite our prediction of functional redundancy of effectors between different mutualistic fungal lineages, the reality of low genomic sequence conservation between effectors still means that we cannot make functional inferences for these proteins. Therefore, it remains to us a mammoth task of characterising these proteins to determine their roles during plant colonisation. Interesting future avenues of research could include determining if mutualistic effectors control aspects of the symbiosis such as hormone homeostasis, carbon movement through membrane interfaces, invertase activity or secondary metabolism.

Outlook

If the effectors characterised to date are any pattern for the activity of other mutualistic effectors the question must be asked: how does the biological activity of effector proteins in some fungal lineages promote a mutualistic lifestyle while in other fungal lineages it promotes pathogenesis? Further, why do the biological targets of mutualistic and pathogenic effectors appear to be so similar? For instance, both pathogenic microorganisms and mutualistic fungi are using effector proteins to manipulate the defenses of plant hosts. By inhibiting the expression of these effectors, mutualistic plant-fungal interactions are aborted just as they are in pathogenic plant-fungal interactions [24**, 25**]. Could it be that the effectors of fungi from both lifestyles target, but oppositely affect, the biological outcome of the same plant pathway? An example in support of this hypothesis would be effectors that target plant JAZ proteins: the mutualistic effector MiSSP7 blocks JAZ degradation and JA signalling *in planta*, while the pathogenic effector HopX1 oppositely affects JAZ protein function and promotes JA signalling [25**, 39*].

Further work needs to occur at the intersection between mutualistic effector biology and pathogenic effector biology to determine if this latter pattern holds true whereby the effectors from fungi of each lifestyle work in opposing manners.

Conclusion

Throughout evolutionary history mutualistic organisms have proven to be close partners of terrestrial plants. If this interaction is based strictly on nutrient exchange dynamics or if it is a highly policed interaction strictly controlled by the biological activities of a squadron of effectors is still up for further research [40]. It also remains to be seen if newly discovered small secreted proteins in the genomes of plants that host mycorrhizal fungi [41] may act to exert control on the fungal partner in a similar manner. Should this latter prove to be true, it would be a major break-through in our understanding of how plants ensure the maintenance of an equal relationship with mutualistic microbes. Large scale international efforts, such as the Mycorrhizal Genomes Initiative, the Fungal Tree of Life, the 1000 Fungal Genomes, and the U.S. DOE Plant-Microbes Interface projects and many other research consortia globally are tackling these difficult questions and we anticipate that their discoveries in the years to come will revolutionise our understanding of mutualistic interactions.

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495

Figure 1. Mycorrhizal fungi express a higher proportion of small effector-like secreted proteins as compared to saprotrophic fungi when grown in the presence of plant tissues.

(A) Percentage of small secreted proteins (SSPs) encoded by each genome that show increased transcript accumulation during symbiosis with plant tissues. An up-regulated transcript is considered as follows: in *Hebeloma cylindrosporum*-*Pinus pinaster* mycorrhiza compared to free-living mycelium; in *Oidiodendron maius*-*Vaccinium myrtillus* mycorrhiza compared to free-living mycelium; in *Paxillus involutus*-*Fagus sylvatica* mycorrhiza compared to free-living mycelium; in *Piloderma croceum*-*Quercus robur* mycorrhiza compared to free-living mycelium; in *Sebacina vermifera*-*Arabidopsis thaliana* mycorrhiza compared to free-living mycelium; in *Suillus luteus*-*Pinus sylvestris* mycorrhiza compared to free-living mycelium; G UP-regulated transcripts in *Tulasnella calospora*-*Serapias vomeracea* mycorrhiza compared to free-living mycelium; in *Serpula lacrymans* mycelium grown on shavings of *Pinus sylvestris* sapwood compared to control mycelium grown on glucose (MMN medium); in *Serpula lacrymans* interacting with roots of *Picea sylvestris* (pseudomycorrhiza) compared to control mycelium grown on glucose (MMN medium); in *Amanita muscaria*-*Populus tremula* x *tremuloides* mycorrhiza compared to free-living mycelium.

(B) Percentage of each genome the code for SSPs. Grey bars indicate mycorrhizal lineages and white bars indicate saprotrophic lineages.