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More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis

Rusty Rodriguez[1,2,*] and Regina Redman[2,3]

[1] US Geological Survey, Seattle, WA 98115, USA

[2] University of Washington, Seattle, WA, USA

[3] Montana State University, Bozeman, MT, USA

Abstract

All plants in natural ecosystems are thought to be symbiotic with mycorrhizal and/or endophytic fungi. Collectively, these fungi express different symbiotic lifestyles ranging from parasitism to mutualism. Analysis of *Colletotrichum* species indicates that individual isolates can express either parasitic or mutualistic lifestyles depending on the host genotype colonized. The endophyte colonization pattern and lifestyle expression indicate that plants can be discerned as either disease, non-disease, or non-hosts. Fitness benefits conferred by fungi expressing mutualistic lifestyles include biotic and abiotic stress tolerance, growth enhancement, and increased reproductive success. Analysis of plant–endophyte associations in high stress habitats revealed that at least some fungal endophytes confer habitat-specific stress tolerance to host plants. Without the habitat-adapted fungal endophytes, the plants are unable to survive in their native habitats. Moreover, the endophytes have a broad host range encompassing both monocots and eudicots, and confer habitat-specific stress tolerance to both plant groups.

Key words: *Colletotrichum*, fungal endophytes, stress tolerance, symbiosis, symbiotic lifestyle.

Introduction

Throughout evolutionary time plants have been confronted with various abiotic and biotic stresses. Lacking any form of locomotion, plants have depended on seed dispersal, vegetative growth, and complex physiology either to escape or to mitigate the impacts of stress. All plants are known to perceive and transmit signals, and respond to stress such as drought, heat, salinity, and disease (Bohnert *et al*., 1995; Bartels and Sunkar, 2005). Some biochemical processes are common to all plant stress responses including the production of osmolytes, altering water movement, and scavenging reactive oxygen species (ROS) (Leone *et al*., 2003; Maggio *et al*., 2003; Tuberosa *et al*., 2003). Although there has been extensive research in plant stress responses, it is not known why so few species are able to colonize high stress habitats. However, plant stress research rarely takes into consideration a ubiquitous aspect of plant biology—fungal symbiosis.

Since the first description of symbiosis (De Bary, 1879), several symbiotic lifestyles have been defined based on fitness benefits to or impacts on host and symbiont (Lewis, 1985). After >100 years of research it is reasonable to conclude that most, if not all, multicellular life on earth is symbiotic with micro-organisms. For example, all plants in natural ecosystems are thought to be symbiotic with mycorrhizal and/or endophytic fungi (Petrini, 1996; Brundrett, 2006). Recent studies indicate that fitness benefits conferred by mutualistic fungi contribute to or are responsible for plant adaptation to stress (Read, 1999; Stone *et al*., 2000; Rodriguez *et al*., 2004). Collectively, mutualistic fungi may confer tolerance to drought, metals, disease, heat, and herbivory, and/or promote growth and nutrient acquisition. It has become clear that at least some plants are unable to endure habitat-imposed abiotic and biotic stresses in the absence of fungal endophytes (Redman *et al*., 2002*b*). Since there are several excellent

* To whom correspondence should be addressed. E-mail: rustyrod@u.washington.edu

reviews on mycorrhizal and endophytic fungi (Carroll, 1988; Read, 1999; Stone *et al.*, 2000; Schardl and Leuchtmann, 2005; Brundrett, 2006), the focus of this discussion will be on two aspects of fungal endophyte biology: symbiotic lifestyle switching (Redman *et al.*, 2001) and the recently observed ecological phenomenon habitat-adapted symbiosis (HA-symbiosis; Rodriguez *et al.*, 2008). It is hypothesized that HA-symbiosis allows plants to establish in high stress habitats.

Fungal endophytes

Unlike mycorrhizal fungi, endophytes reside entirely within host tissues and emerge during host senescence. These fungi comprise a phylogentically diverse group that are members of the dikarya (Carroll, 1988; Schardl and Leuchtmann, 2005; Van Bael *et al.*, 2005; Girlanda *et al.*, 2006; Arnold and Lutzoni, 2007). While most endophytes belong to the Ascomycota clade, some belong to the Basidiomycota. Although these fungi are often grouped together, they can be discriminated into different functional groups just as has been done with mycorrhizal fungi (Brundrett, 2006). Currently, endophytes can be sub-divided into four classes based on host range, colonization pattern, transmission, and ecological function (Rodriguez *et al.*, in review). Nevertheless, endophytes have been shown to confer fitness benefits to host plants including tolerance to herbivory, heat, salt, disease, and drought, and increased below- and above-ground biomass (Bacon and Hill, 1996; Clay and Holah, 1999; Sahay and Varma, 1999; Redman *et al.*, 2001, 2002*b*; Arnold *et al.*, 2003; Waller *et al.*, 2005; Márquez *et al.*, 2007).

The symbiotic continuum, lifestyle switching, and host range

Collectively, fungi express several different symbiotic lifestyles that are defined by fitness benefits to plant hosts and symbionts (Lewis, 1985). The range of symbiotic lifestyle expression from mutualism to parasitism has been described as the symbiotic continuum (Carroll, 1988; Johnson *et al.*, 1997; Saikkonen *et al.*, 1998; Schulz *et al.*, 1999; Schardl and Leuchtmann, 2005). Within each group of fungal symbionts there are isolates and/or species that span the symbiotic continuum by expressing different lifestyles. For example, the endophyte genus *Epichloe* comprises species that express either mutualistic or parasitic lifestyles (Schardl and Leuchtmann, 2005). Several studies that focused on the isolation of endophytes from asymptomatic plant tissues indicate that individual species express either mutualistic, commensal, or parasitic lifestyles when re-inoculated back on the original host species (Schulz *et al.*, 1999). This indicates that both mutualists and pathogens infect plants and remain quies-

cent until plant senescence. This represents an excellent ecological strategy for fungi to capitalize on plant nutrients. By already being established in tissues, endophytes have immediate access to plant nutrients made available during plant senescence.

Studies on host genotype versus symbiotic lifestyle expression revealed that individual isolates of some fungal species could span the symbiotic continuum by expressing either mutualistic or pathogenic lifestyles in different host plants (Redman *et al.*, 2001). For example, *Colletotrichum* species are classified as virulent pathogens, yet several species can express mutualistic lifestyles in non-disease hosts (Table 1). Mutualistic benefits conferred by *Colletotrichum* spp. include disease resistance, growth enhancement, and/or drought tolerance (Redman *et al.*, 2001). Although the genetic basis of symbiotic communication is not yet known, subtle differences in host genomes have profound effects on the outcome of symbiotic interactions. For example, commercially grown tomato (*Solanum lycopersicum*) is known to possess relatively few genetic differences between varieties yet is able to express high levels of phenotypic plasticity (Miller and Tanksley, 1990; Tanksley, 2004; Brewer *et al.*, 2007). When *C. magna* is introduced into different tomato cultivars, the fungus may express either mutualistic, commensal, or parasitic lifestyles. While parasitic and mutualistic lifestyles are easily observed, commensal lifestyles are often designated when no host fitness benefit is observed. However, depending on the traits being assessed, the commensal designation may be misleading. For example, *C. gloeosporioides* was designated a pathogen of strawberry and a commensal of tomato because it conferred no disease protection (Redman *et al.*, 2001). However, *C. gloeosporioides*

Table 1. *Symbolic lifestyle expression of* Colletotrichum *species versus plant host*

Fungal pathogen	Disease host[a]	Non-disease host[b]	Lifestyle expressed	
			Disease stress[c]	Drought stress[d]
C. magna	Watermelon	Tomato	Mutualism	Mutualism
C. musae	Banana	Pepper	Mutualism	Mutualism
C. orbiculare	Cucumber	Tomato	Mutualism	Mutualism
C. acutatum	Strawberry	Watermelon	Commensalism	Mutualism
C. gloeosporioides	Strawberry	Watermelon	Commensalism	Mutualism

[a] Species were isolated from disease lesions on the indicated host plants.
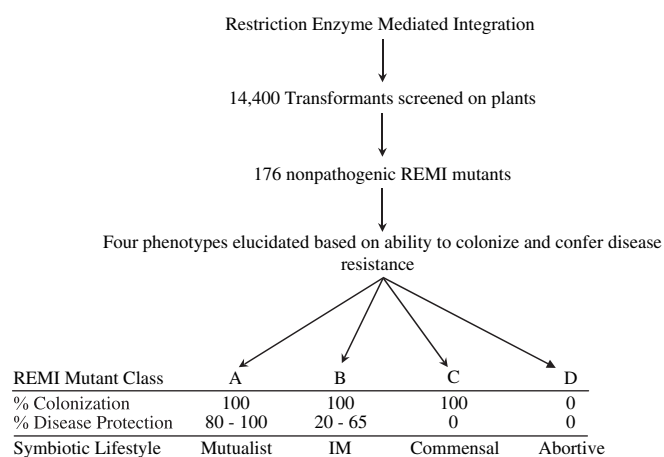[b] Host plants that are asymptomatically colonized by the respective *Colletotrichum* spp.
[c] Symbiotic lifestyle expressed after asymptomatic colonization. Lifestyles were defined by the ability of each *Colletotrichum* sp. to confer disease resistance against virulent *Colletotrichum* pathogens of the non-disease hosts (data from Redman *et al.*, 2001).
[d] Symbiotic lifestyle expressed after asymptomatic colonization. Lifestyles were defined by the ability of each *Colletotrichum* sp. to confer drought tolerance based on the length of time before wilting after cessation of watering (data from Redman *et al.*, 2001).

increased plant biomass and conferred drought tolerance to tomato plants, and was therefore designated a mutualist.

A series of experiments were performed to characterize the genetic basis of fungal symbiotic lifestyles. UV mutagenesis of a virulent isolate (CmL2.5) of *C. magna* resulted in the isolation of a non-pathogenic mutant (Path-1) that was able to colonize host plants asymptomatically (Freeman and Rodriguez, 1993). Path-1 conferred several fitness benefits to hosts, including disease and drought resistance, and growth enhancement. Based on these fitness benefits, it was concluded that Path-1 was expressing a mutualistic lifestyle in host plants. Additional studies involving gene disruption by restriction enzyme-mediated integration (REMI) with a selectable plasmid resulted in the generation of non-pathogenic mutants that differed in the ability to confer disease resistance (Fig. 1; Redman *et al.*, 1999). The UV and REMI mutants lost the ability to switch between lifestyles and were 'locked' into one lifestyle (either mutualism, intermediate-mutualism, or commensal). These results indicate that the ability to switch between symbiotic lifestyles, at least in this species, is controlled by single genetic loci.

Although the original experiments on lifestyle switching were performed with *Colletotrichum* species known to be pathogenic, similar results have been observed with other endophytes from plants in natural habitats (RY Rodriguez, unpublished results). What does this mean with regard to host specificity? It appears that there are non-hosts that a fungus is unable to infect and two types of hosts that fungi can colonize: disease hosts that they parasitize and non-disease hosts that they asymptomatically colonize.

Colonization of non-disease hosts by pathogenic *Colletotrichum* species is asymptomatic and there are no observable differences between colonized and uncolonized plants in the absence of stress, unless the endophyte promotes plant growth (Redman *et al.*, 1999, 2002*a*). Conventional views suggest that pathogens either cause disease or induce host defence systems which terminate the infection process. When *Colletotrichum* species express mutualistic lifestyles and confer disease resistance, host defence systems are not activated unless the symbiotic plants are challenged with a virulent pathogen (Redman *et al.*, 1999, 2002*a*). Once challenged, the host defence systems activate very rapidly (<24 h) to maximal levels (Redman *et al.*, 1999).

The ability to switch lifestyles brings up some interesting questions:

(i) Is there an evolutionary direction to symbiotic lifestyles? Clavicipitaceous endophytes expressing mutualisms are hypothesized to have evolved directionally from pathogenic ancestors (Schardl and Leuchtmann, 2005). The situation with at least some other endophytes appears to be quite different, where the evolution of symbiotic lifestyle appears to lack specific directionality (Arnold and Lutzoni, 2007). Endophytes that can switch lifestyles may represent evolutionary transitions or simply fungi that have achieved a higher degree of ecological flexibility to ensure optimal growth and reproduction in a variety of hosts.

(ii) Do plants inadvertently participate or possibly instigate disease processes? Individual fungal isolates can equally colonize different plants irrespective of the symbiotic lifestyle they express. For *C. magna* to express mutualism in one tomato cultivar and parasitism in another suggests that disease may reflect miscommunication rather than aggressive pathogenicity.

Restriction Enzyme Mediated Integration

↓

14,400 Transformants screened on plants

↓

176 nonpathogenic REMI mutants

↓

Four phenotypes elucidated based on ability to colonize and confer disease resistance

REMI Mutant Class	A	B	C	D
% Colonization	100	100	100	0
% Disease Protection	80 - 100	20 - 65	0	0
Symbiotic Lifestyle	Mutualist	IM	Commensal	Abortive

Fig. 1. Gene disruption (restriction enzyme-mediated integration, REMI) of fungal symbiotic lifestyle loci in *Colletotrichum magna*. Symbiotic lifestyles reflect the ability of REMI mutants to colonize host plants (watermelon) asymptomatically and confer disease resistance against the virulent wild type. REMI mutants were designated either as mutualists, intermediate mutualists, or commensals based on disease protection, or abortive if they were unable to colonize host tissues. Although these lifestyle designations reflect quantitative differences, they probably reflect a continuum of symbiotic lifestyles represented among the mutants. Methods and data are from Redman *et al.* (1999*a*).

Symbiosis and stress tolerance

There are numerous reports of fungal symbionts conferring tolerance to stress to host plants, including herbivory, drought, heat, salt, metals, and disease (Bacon and Hill, 1996; Clay and Holah, 1999; Sahay and Varma, 1999; Redman *et al.*, 2001, 2002*b*; Arnold *et al.*, 2003; Waller *et al.*, 2005; Márquez *et al.*, 2007; Rodriguez *et al.*, 2007). It is interesting that the stress tolerance conferred by some endophytes involves habitat-specific fungal adaptations. For example, within the geothermal soils of Yellowstone National Park, a small number of plant species reside. One plant species (*Dichanthelium lanuginosum*) has been studied and found to be colonized by one dominant endophyte (*Curvularia protuberata*). *Curvularia protuberata* confers heat tolerance to the host plant, and neither the fungus nor the plant can survive separate from one another when exposed to heat stress >38 °C (Redman

et al., 2002*b*). The ability of the endophyte to confer heat tolerance requires the presence of a fungal RNA virus (Márquez *et al.*, 2007). While the genetic/biochemical role of the virus in symbiotically conferred heat tolerance is not known, it is surmised that the virus is providing biochemical functionality to the fungus and it is not the virus that directly confers heat tolerance. A comparison of *C. protuberata* isolates from geothermal and non-geothermal plants revealed that the ability to confer heat tolerance was specific to isolates from geothermal plants (Rodriguez *et al.*, 2008). Therefore, the ability to confer heat tolerance is a habitat-adapted phenomenon.

Another example of habitat-specific fungal adaptation involves a native dunegrass (*Leymus mollis*) on coastal beaches of Puget Sound, WA (Rodriguez *et al.*, 2008). *Leymus mollis* is colonized by one dominant fungal endophyte (*Fusarium culmorum*) that can be isolated from above- and below-ground tissues and seed coats. *Fusarium culmorum* confers salt tolerance to the host plant which cannot survive in coastal habitats without the habitat-adapted endophyte. A comparison of *F. culmorum* isolates from *L. mollis* and a non-coastal plant revealed that the ability to confer salt tolerance was specific to isolates from the coastal plants, indicating that the ability to confer salt tolerance is a habitat-adapted phenomenon (Rodriguez *et al.*, 2008).

A comparison of *C. protuberata*, *F. culmorum*, and *C. magna* isolates further supports habitat-specific adaptation of endophytes: *C. protuberata* confers heat but not disease or salt tolerance; *F. culmorum* confers salt but not heat or disease tolerance; and *C. magna* confers disease but not heat or salt tolerance (Rodriguez *et al.*, 2008). These symbiotically conferred stress tolerances conform to the evolutionary dynamics that must play out in the different habitats, with fungi adapting to habitat-specific stresses and conferring stress tolerance to host plants. This habitat-specific adaptation is defined as HA-symbiosis, and it is hypothesized that this allows plants to establish and survive in high stress habitats.

Biochemical basis of endophyte-conferred stress tolerance

It is fascinating that after 400 million years of evolution there are plants that require symbiotic associations for stress tolerance. There has been an enormous research effort in plant stress physiology that is described in several excellent books and reviews. Although previous studies have elucidated how plants respond to stress, they rarely consider symbiotic contributions.

Symbiotically conferred disease tolerance appears to involve different mechanisms depending on the endophyte. For example, the ability of a non-pathogenic *Colletotrichum* mutant (Path-1 that expresses a mutualism) to confer disease resistance is correlated to a rapid and

Table 2. *Physiological defence activity versus symbotically conferred disease conferred disease resistance by* Colletotrichum magna

Methods and physiological data are from Redman *et al.* (1999).

Host	Peroxidase activity[a]		PAL activity[b]		Lignin deposition[c]	
	24 h	48 h	24 h	48 h	24 h	48 h
Watermelon (E–)[d]	2.76	3.46	2.27	2.90	–	+
Watermelon (E+)[e]	5.77	6.30	2.50	3.70	+++	++++
Cucumber (E–)	0.63	1.31	0.02	0.25	–	+
Cucumber (E+)	1.80	2.34	.27	0.34	+++	++++

[a] Activity based on a guaiacol/H_2O_2 assay, and units indicate change in A_{470} min^{-1} µg^{-1} protein.
[b] Activity based on the production of cinnamic acid, and units indicate change in A_{290} min^{-1} µg^{-1} protein.
[c] Qualitative assessment of the absence (–) or presence (+) of lignin visualized with acidic phloroglucinol.
[d] (E–)=endophyte (*C. magna*) free.
[e] (E+)=endophyte (*C. magna*) colonized.

strong activation of biochemical processes known to confer resistance (Redman *et al.*, 1999). In the absence of pathogen challenge, Path-1-colonized plants do not appear to activate host defence systems. However, when Path-1-colonized watermelon and cucumber seedlings were exposed to a virulent pathogen, peroxidase and phenylalanine ammonia lyase activity and lignin deposition increased within 24 h to levels that non-symbiotic plants never achieved (Table 2; Redman *et al.*, 1999). Interestingly, *Colletotrichum*-conferred disease resistance is localized to tissues that the fungus has colonized, and is not systemic. The results suggest that the endophyte may be acting as a type of biological trigger that activates host defence systems. The fact that *Colletotrichum* spp. expressing non-pathogenic lifestyles do not activate host defence in the absence of pathogen challenge may be viewed as either suppression of host defences or eluding host recognition. However, the dynamics of host defence activation suggest that the endophytes are recognized and do not suppress defence systems.

In barley, the root endophyte *Piriformospora indica* confers disease resistance by a different mechanism. Symbiotic plants are thought to resist necrotrophic root pathogens due to increased activity of glutathione–ascorbate antioxidant systems (Waller *et al.*, 2005). Unlike *Colletotrichum* endophytes, disease resistance conferred by *P. indica* appears to be systemic. It is not clear if *P. indica* increases antioxidation systems in the absence of pathogens or if other aspects of host physiology are involved in resistance.

The differences between *Colletotrichum* spp.- and *P. indica*-conferred disease resistance may indicate that a greater diversity of mechanisms may yet be elucidated. Regardless, these results warrant a more comprehensive analysis of endophyte-conferred disease resistance.

Symbiotic plasticity and fungal taxonomy

One deficiency in species designations is a dearth of functional ecological descriptions, symbiotic lifestyle potential, and host ranges. A good example of this issue is the fact that *C. protuberata* is described as a plant pathogen of several monocots (Farr *et al.*, 1989). Yet, *C. protuberata* isolate Cp4666D is a mutualist in *Dichanthelium lanuginosum*, conferring heat and drought tolerance (Rodriguez *et al.*, 2008). While *Curvularia* species are not known to have broad disease-host ranges, *C. protuberata* from the monocot *D. lanuginosum* is a mutualist (confers heat tolerance) in the eudicot tomato, and isolates from non-geothermal plants do not confer heat tolerance (Márquez *et al.*, 2007; Rodriguez *et al.*, 2008). A similar scenario occurs with *F. culmorum.* Designated as a virulent plant pathogen, *F. culmorum* causes disease on a variety of crop plants (Farr *et al.*, 1989). However, the *F. culmorum* isolate FcRed1 from dunegrass is a mutualist in dunegrass and tomato conferring salt tolerance, and isolates from non-coastal plants do not confer salt tolerance (Rodriguez *et al.*, 2008). These examples indicate that the current concept of a fungus being categorized as either a pathogen, saprophyte, or mutualist is inadequate to address the fact that individual species can represent significant ecological plasticity.

The ability of 'pathogenic' *Colletotrichum* species to switch symbiotic lifestyles and express mutualisms provides insight into why these species are so ubiquitous. It has been suggested previously that pathogens may be present in non-disease host plants constituting potential inocula for disease. In fact, *C. acutatum* asymptomatically colonizes pepper, eggplant, bean, and tomato plants, which can subsequently provide inoculum for disease outbreaks in strawberry plants (Freeman *et al.*, 2001). So, at least in this genus, species may move freely between lifestyles and hosts, thereby expanding bio-geographic distribution. It is unlikely that this phenomenon is specific to *Colletotrichum* as asymptomatic colonization of hosts has been reported for other genera such as *Fusarium* (Bacon and Yates, 2006).

Incorporating information on lifestyle expression and ecological functionalities would allow ecologists to understand better the role of fungi in ecosystem processes, geneticists to understand better genome differences between isolates, and mycologists to understand phenotypic and ecological plasticity.

Symbiotic communities

While this discussion has focused on fungal symbionts, it is important to point out that plants represent communities of fungi, bacteria, viruses, and/or algae. All of these micro-organisms contribute to the outcome of symbiosis and hence increase the complexity of studying plant biology. Moreover, fungal symbionts may also harbour bacteria and viruses that can have dramatic effects on symbiotic communication. For example, the class 2 endophyte *C. protuberata* (Cp4666D), originally isolated from plants growing in geothermal soils, contains a double-stranded RNA (dsRNA) virus that is required for symbiotically conferred heat tolerance (Márquez *et al.*, 2007). In the absence of the virus, Cp4666D asymptomatically colonizes plants but confers no heat tolerance. Therefore, a three-way symbiosis (a virus in a fungus in a plant) is required for thermal tolerance. This was an unexpected result and reflects our limited understanding of symbiotic systems and how they function. More importantly, it indicates the need to study plants from a symbiotic systems perspective to elucidate the contributions of all symbionts.

Summary

Both laboratory and field studies have demonstrated that at least some plant species in natural habitats require fungal endophytes for stress tolerance and survival. Since colonizing land ~400 million years ago, plants have evolved intragenomic mechanisms to perceive and transmit signals, and respond to stress (Bohnert *et al.*, 1995; Bartels and Sunkar, 2005), but most plants lack the adaptive capability to mitigate the impacts of stress (Alpert, 2000). At least some plants depend on intergenomic epigenetic processes provided by symbiotic fungi for stress adaptation. The observations described in this manuscript raise some fundamental questions in plant biology. Why have plants in high stress habitats not evolved intragenomic capabilities for stress adaptation? Can plants adapt to stress without symbiotic involvement? Why are so few plants adapted to high stress habitats? Answers to these questions will require extensive research efforts over the coming decades and are necessary before ecosystem processes are fully understood.

Acknowledgements

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