Were endophytes pre-adapted for defensive mutualism?

M.S. TORRES^{1*}, A.P. SINGH^{1,2}, N. VORSA^{2,1}, T. GIANFAGNA¹ and J.F. WHITE Jr.¹

¹Department of Plant Biology and Pathology, Rutgers University, 59 Dudley Road, New Brunswick, New Jersey 08901

²Marucci Blueberry-Cranberry Research Center, Rutgers University, 125a Lake Oswego Road, Chatsworth, NJ 08019

*mstorres2001@yahoo.com, mstorres@eden.rutgers.edu

Abstract

Defensive mutualism in many grass endophytes involves herbivore deterrence, enhanced disease suppression and drought tolerance. According to our current understanding of the mechanisms, these defensive effects are unique features of the fungi and specific fungal qualities are responsible for each. We examined a selection of species of family Clavicipitaceae for defensive traits including ergot alkaloids and loline production and development of endophytic and epiphytic mycelial states. When features responsible for defensive effects are mapped onto phylogenetic trees showing relationships in the family, it is apparent that all defensive features of endophytic fungi pre-existed evolution of endophytism. We suggest that once endophytism evolved, the alkaloids and other fungal-mediated defensive features switched from previous functions to serve host defense functions. Defensive mutualism in the Clavicipitaceae endophytes naturally evolved through use of pre-existing fungal features after the step to endophytism was made.

Keywords: Clavicipitaceae, defensive mutualism, endophytes, ergot alkaloids, loline alkaloids

Introduction

Defensive mutualism in the Clavicipitaceae (Hypocreales, Ascomycota) is a concept that was first articulated by Keith Clay to explain deterrent effects of endophytes primarily against insects. Based on experiments that demonstrated that insects show a feeding preference for endophyte-free grasses, it was proposed that defense of the host plant from herbivores was an important ecological function of endophytes (Clay 1988). Observations on certain endophyte-infected grasses also suggested that endophytes protected grasses from herbivores in general, such as prairie voles (*Microtus ochrogaster*) (Durham & Tannenbaum 1998) and avian passerines (Madej & Clay 1991). In vertebrates grazing endophyte-infected tall fescue and perennial ryegrass, toxicoses including “fescue foot” and “ryegrass staggers” are common occurrences. Secondary metabolites produced by the endophytes are responsible for anti-herbivore effects. These metabolites include ergot alkaloids, lolines, peramine and other metabolites, not all of which have been fully characterised (Porter 1994).

Our understanding and interpretation of defensive mutualism as it pertains to endophytes in Clavicipitaceae has become more elaborate as a more complete knowledge of endophyte effects on grass hosts has developed. The defensive mutualism concept was expanded to include defense of the host from abiotic effects when it became clear that some endophytes imparted enhanced tolerance to drought (West *et al.* 1990; Malinowski & Belesky 2000). More recently it has been proposed that certain endophytes also benefit hosts by defending them from some foliar pathogens. Clarke *et al.* (2006) showed that in the fine and hard fescues endophyte-mediated suppression of the leaf spot disease “dollar spot” caused by the fungus *Sclerotinia homeocarpa* is commonly observed. In addition, Bonos *et al.* (2005) also have shown endophyte-mediated resistance to “red thread disease” caused by *Laetisaria fuciformis* in fine fescues. From an

evolutionary perspective it is of interest to evaluate whether the defensive features of grass endophytes evolved *de novo* when the endophytes evolved or instead pre-existed evolution of the grass endophytes and were present in species that were ancestral to the *Epichloë/Neotyphodium* endophyte.

Our hypothesis is that endophytes in Clavicipitaceae were pre-adapted for defensive mutualism symbiosis with host plants. The characteristics we attribute to defense of hosts pre-existed any host defensive function. Once endophytism evolved the pre-adaptive characteristics of the fungi naturally gave way to defensive mutualisms now evident among endophytic species. To illustrate this argument we analysed and placed in a phylogenetic context each of the beneficial functions independently, alkaloid production (ergot alkaloids and loline alkaloids), endophyte-mediated disease suppression and enhanced drought tolerance.

Materials and Methods

Phylogenetic analysis

To analyse phylogenetic relationships LSU rDNA sequences of a selection of species representing the family Clavicipitaceae were selected (Table 1), aligned and analysed using the program PAUP v 4.0 (Swofford 2002) by maximum likelihood and Bayesian analysis. ModelTest v.3.06 (Posada & Crandall 1998) was used to select the best fitting model of sequence evolution determined by Akaike information criterion (Akaike 1974).

The model selected was TRN with proportion of invariable sites (I) and gamma distribution (G). The parameters include base frequencies A=0.2628, C=0.2197, G=0.3375, T=0.1799; rate matrix [A-C]=1.0, [A-G]=2.0369, [A-T]=1.0, [C-G]=1.0, [C-T]=10.1208; [G-T]=1.0; I=0.4879 and G=0.5224 and this model was incorporated into PAUPv.4.0. The most likely tree (-ln 3877.97) is shown in Fig. 1. Bayesian inference was used to estimate branch support (posterior probability) under likelihood using Mr Bayes 3.0 (Huelsenbeck 2000). Bayesian analysis was run three times with four mcmc (Markov Chain Monte Carlo) chains for 1,000,000 generations, sampling every 100 generations. The 30,000 trees resulting from the three runs were pooled and 28,500 were imported into PAUP to construct a majority rule consensus tree after discarding the asymptotic trees (burn in).

Alkaloids analyses

Twelve species were subjected to alkaloid analysis (Table 1). For ergot alkaloid analysis fungal extracts were subjected to high performance liquid chromatography (HPLC) and mass spectrophotometry (LC-MS) analyses following procedures of Torres *et al.* (2006). For loline analysis, fungal extracts were subjected to gas chromatography-mass spectroscopy (GC-MS) analysis (Blankenship *et al.* 2001; Yates *et al.* 1990). The results were mapped into a phylogenetic tree (Fig. 1).

Results and Discussion

Alkaloid production and herbivore deterrence

Secondary metabolites produced in grass-endophyte associations are generally assumed to be defensive in function (Panaccione

Figure. 1 The most likely tree ($-\ln(-\ln 3877.97)$) as determined by Paup using TrN+I+G model of evolution. Numbers on the branches indicate posterior probabilities as a percentage for the node they proceed (only $\geq 50\%$ shown). (Symbols, + ergot alkaloids, * lolines).

& Schardl 2003). Generally an array of alkaloids and other secondary metabolites are produced by the endophyte in each symbiotic association suggesting a spectrum of biological activities (Panaccione 2005). It is also reasonable to assume that there are synergisms of these compounds with one another. Ergot alkaloids (ergonovine and derivatives) and lolitremes have been associated with vertebrate toxicity (Schardl & Philips 1997). Ergot alkaloids have also been shown to deter feeding by mammals (Panaccione *et al.* 2006).

Lolines and possibly peramine have been mainly associated with insect toxicity (Porter 1994). It has been shown that aphids (*Rhopalosiphum padi*), Japanese beetle (*Popillia japonica*), fall

armyworm (*Spodoptera frugiperda*), and several other insects are deterred from feeding by lolines (Wilkinson *et al.* 2000; Riedell *et al.* 1991; Patterson *et al.* 1991).

Because *Dussiaella*, *Hyperdermium* and *Hypocrella* are deeply rooted epibionts in phylogenetic analyses (see Fig. 1) and they also possess ergot alkaloids it is evident that ergot alkaloids are a feature that pre-existed the evolution of endophytism and defensive mutualism with the host plant.

Function switching

It is unlikely that alkaloids in the non endophytic species of Clavicipitaceae play any role in defense of their host plants.

Table 1 Large subunit ribosomal DNA sequences included in phylogenetic analysis and alkaloid analysis

Taxa	LSU	Alkaloid
<i>Ascoplyporus polychrous</i> Möller	AY886547	RUTTP-JB211
<i>Balansia brunnans</i> Lewis & White	AY3207046	-
<i>Balansia epichloë</i> (Weese) Diehl	U68121	-
<i>Atkisonella hypoxylon</i> (Peck) Diehl	U57087	-
<i>Balansia henningsiana</i> (Möller) Diehl	U57678	-
<i>Balansia nigricans</i> Diehl	-	RUTTP-BN1
<i>Balansia obtecta</i> Diehl	U68120	-
<i>Balansia strangulans</i> (Mont.) Diehl	U57679	CBS334.96
<i>Atkinsonella texensis</i> (Diehl) Leuchtm. & Clay	U68113	-
<i>Balansia pilulaeformis</i> (Berk. & M.A. Curtis) Diehl	AF543788	-
<i>Chaunopycnis alba</i> Gams	AF245296	RUTTP-GB1
<i>Claviceps africana</i> Freder., Mantle & De Milliano	AF245294	-
<i>Claviceps fusiformis</i> Loveless	U17402	-
<i>Claviceps paspali</i> Stevens & J.G. Hal	U47826	-
<i>Claviceps purpurea</i> (Fr.) Tul	U57085	sclerotia
<i>Claviceps ranunculoides</i> Möller	AF245295	-
<i>Cordyceps capitata</i> (Holmsk.) Link	U57086	-
<i>Cordyceps militaris</i> (L.) Link	AF043135	-
<i>Cordyceps ophioglossoides</i> (Ehrh.) Link	U47827	-
<i>Cordyceps spagazzinii</i> Torres <i>et al.</i>	DQ196435	MYA3684
<i>Dussiella tuberiformis</i> (Berk. & Ravenel) Pat	U57083	MYA 2810
<i>Epichloë amarillans</i> White	U57680	-
<i>Epichloë typhina</i> (Pers.:Fr.) Tul.	U17396	-
<i>Hyperdermium bertonii</i> White <i>et al.</i>	AF242354	-
<i>Hyperdermium pulvinatum</i> White <i>et al.</i>	AF242353	RUTTP-HP1
<i>Hypocrella panamensis</i> Torres <i>et al.</i>	DQ127242	RUTTP-JB207
<i>Hypocrella nectrioides</i> Thaxt	U47832	-
<i>Hypocrella phyllogena</i> (Mont.) Speg	AY518372	RUTTP-4iso8
<i>Lecanicillium lecanii</i> (Zimmermann) Zare <i>et Gams</i>	U17414	-
<i>Leuconectria clusiae</i> (Samuels & Rogerson) Rossman <i>et al.</i> U17412	-	
<i>Nectria cosmariospora</i> Ces. & De Not.	U17407	-
<i>Nectria radicola</i> Gerlach & L. Nilsson	U17415	-
<i>Neotyphodium coenophialum</i>	AF160241	-
<i>Paecilomyces lilacinus</i>	-	GB-5470
<i>Torrubiella piperis</i> Bischoff & White	AY466442	-
<i>Torrubiella wallacei</i> Evans	AY184967	-
<i>Torrubiella luteorostrata</i> Zimm.	AY554252	-
<i>Torrubiella superficialis</i> Zimm.	-	RUTTP-SU70

This is because these fungi are not systemic in host plants and alkaloids likely remain within the restricted superficial mycelia. Their functions in the superficial fungal mycelia are unknown and it could be assumed that they protect the stromata from insect herbivory (Torres *et al.* unpublished). Regardless of their function, it is apparent that epibiotic species in the Clavicipitaceae were pre-adapted for defensive mutualism by production of compounds that now serve to deter herbivore feeding. We propose that once the fungi evolved as systemic endophytes, ergot alkaloids switched function from “self defense” of stromata to “host defense”.

Lolines were detected in species including *Hyperdermium pulvinatum* and *Hypocrella phyllogena* (Fig. 1). Both these species are epibiotic and more deeply rooted in the phylogenetic analysis

than endophytic species of *Epichloë* and *Balansia*. It seems likely that lolines also occur in additional species of Clavicipitaceae, although we did not detect it. Previous work with lolines suggests that it is infrequently expressed in culture (Blankenship 2004). The discovery of lolines in other more deeply rooted members of the family suggests that the defensive compound loline pre-existed evolution of the endophytes and may be a compound that is common in the family.

Another alkaloid that is important in the endophyte defensive mutualism is peramine. This compound is believed to be detected by some insects that feed on the aboveground grass plants. Future studies will be needed to evaluate whether peramine was newly evolved in the endophytes or is another feature that is common in the family Clavicipitaceae.

Enhanced fungus disease resistance

Endophytes in fine and hard fescues where enhanced disease resistance has been demonstrated also possess an epibiotic or epiphyllous phase where mycelium forms a dense network on surfaces of leaf blades. The superficial mycelium on leaf blades was termed "defensive nets" by Moy *et al.* (2000). In addition to fine and hard fescue, epiphyllous mycelia have been found on numerous additional endophyte infected grasses including *Agrostis hiemalis*, *Poa rigidifolia* and *Hordeum brevisubulatum* (White *et al.* 1996; Dugan *et al.* 2002).

Circumstantial evidence suggests that endophyte-mediated resistance to fungal disease is due to a competitive exclusion phenomenon. Epiphyllous mycelia on leaf blades occupy a niche that many pathogens must colonize prior to entering leaves to incite disease. The plant epiphytic condition is more primitive or more deeply rooted than the endophytic condition in family Clavicipitaceae (See Fig. 1). Scale insect parasite/plant epibiont species (including *Hyperdermium*, *Dussiella* and *Torrubiella*) are among the most deeply-rooted in phylogenetic analysis (Fig. 1). These species infect scale insects, degrade them and live biotrophically as epiphytes that are nourished by nutrients that leak to the plant surface through the stylet wound (Koroch *et al.* 2004). It seems probable that such epiphytic states were ancestral to acquisition of endophytism in genera such as *Epichloë* and *Balansia*. In the *Balansia* clade, the epiphytic species *B. hypoxylon*, and *B. texensis* are more deeply rooted than endophytic species *B. henningsiana*, *B. strangulans* and *B. nigricans*. The epibiotic species in *Balansia* grow superficially on the meristematic surfaces of plants. The epiphyllous condition thus pre-existed acquisition of endophytism in the family.

The endophytes that retain an epiphyllous state tend to be those that grow aggressively and colonise developing leaves internally and externally. These are frequently endophytes that retain the capacity to sexually reproduce using stromata but some that do not produce stromata also develop epiphyllous mycelia. The endophytes of *Neotyphodium lolii* and *N. coenophialum* in perennial ryegrass and tall fescue, respectively, do not produce epiphyllous mycelial states and have not shown any disease resistance to leaf spot diseases. The endophytes retaining the primitive epiphyllous feature are the ones that demonstrate fungus disease resistance. The epiphytic condition of the plant biotrophic epibionts pre-adapted the endophytes for defense against some plant pathogens.

Physiological mechanisms: drought tolerance

Little is clearly understood regarding the mechanisms involved in drought tolerance. One hypothesis of the mechanism involves osmotic adjustment of the meristematic tissues where endophytes reside in the intercellular spaces (Elmi & West 1995). In this phenomenon it has been suggested that osmotically active metabolites such as water-soluble sugars, mannitol, and amino acids like proline and alkaloids (loline) accumulate in intercellular spaces of the meristems and cause a pre-acclimation to drought conditions (Richardson *et al.* 1992; Richardson 2000).

If the osmotic adjustment hypothesis for enhanced drought tolerance is correct then the endophytes may also have been pre-adapted for osmotic adjustment of host meristems by production and secretion of alkaloids and release of other metabolites. Endophytes may also cause some release or leakage of nutrients from host cells and this may contribute to the osmotic adjustment effects.

Conclusions

Our analysis suggests that fungal features responsible for defensive

mutualism in the grass endophytes evolved prior to evolution of endophytism in genera *Balansia*, *Epichloë* and *Neotyphodium*. This suggests that the family was pre-adapted to develop defensive mutualisms once systemic endophytisms evolved. It is apparent that after evolution of endophytism, compounds, physiological processes, and mycelial structures switched from whatever were their previous functions to defense of the host plant.

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