Protective Grass Endophytes

Where are they from and where are they going?

Perhaps the most widely used agents of biological plant protection are endophytic fungal symbionts (endophytes) of forage and turfgrasses. These are fungi of the family *Clavicipitaceae*, which grow between host cells in vegetative tissues, ovules, and seeds of systemically infected grass plants. The existence of these endophytes was not fully appreciated until recent years, although the protection they provide against insect damage (Fig. 1) and drought contributes to the superior agronomic qualities of favorite pasture grasses in North America, Australia, and New Zealand. Unfortunately for livestock farmers, these endophytes also provide a degree of protection from grazing mammals. In 1977, Bacon et al. (2) reported that the grass *Festuca arundinacea* var. *genuina* Schreb. (hexaploid tall fescue) had a fungal endophyte related to *Epichloë typhina* (Pers.:Fr.) Tul., and that this endophyte—now known as *Neotyphodium coenophialum* (Morgan-Jones & Gams) Glenn, Bacon, & Hanlin—was responsible for toxicosis suffered by livestock grazing the grass.

Epichloë species were known for many decades (44), but reports relating that nonpathogenic endophytes could be detrimental to livestock provided new impetus for intensive studies, making the grass–endophyte associations among the best characterized symbioses in biology. Less than two decades of research have yielded a rich body of knowledge about these symbioses:

their secondary product chemistry, ecology, evolution, genetics, and molecular biology; their ecological roles as protectants from insect and vertebrate herbivores, pathogenic fungi and nematodes, and drought; and their effects on host growth and competitiveness. The endophytes produce numerous alkaloids, some of which are unrelated to any known from plants or other fungi. Their genetic and evolutionary complexity is extraordinary. They were the first fungi genetically documented as interspecific hybrids (47,53). Meanwhile, molecular genetic techniques were applied to the endophytes of tall fescue and other grasses, bringing us closer to the prospect of reducing or eliminating their toxicosis to livestock while continuing to employ their bioprotective qualities.

Benefits of Endophytes

The most widely known of the *Epichloë* Tul. species are the *Acremonium* endophytes (Table 1), which as asexual fungi are now classified in form genus *Neotyphodium* Glenn, Bacon, & Hanlin (17) (until recently as *Acremonium* Link sect. *albo-lanosa* Morgan-Jones & Gams). *Neotyphodium* species inhabit tall fescue, perennial ryegrass (*Lolium perenne* L.), and many cool-season grasses with which they are so intimately symbiotic that grass and fungus together act much like a single organism. The asexual endophytes can disseminate only by infecting seeds as they develop on infected mother plants. So, for example, seeds and seedlings of tall fescue Kentucky 31, symbiotic with *N. coenophialum* (= *Acremonium coenophialum* Morgan-Jones & Gams) can arise only from infected mother plants.

It seems unlikely that grasses would continue to harbor asexual endophytes in nature if they were of no benefit (8). In fact, the endophytes have been shown to confer profoundly enhanced fitness to grass hosts (Fig. 1). The range of endophyte benefits is also astounding. The most intensely studied symbioses are tall fescue with *N. coenophialum* and perennial ryegrass with *Neotyphodium lolii* (Latch,

Fig. 1. Effects of *Neotyphodium coenophialum* on tall fescue: (A) Tall fescue infected with *N. coenophialum* and growing on a reclaimed strip mine in eastern Kentucky. (B) The same cultivar without endophyte growing on an adjacent plot. (C) Aphids (*Rhopalosiphum padi*) feeding preferentially on endophyte-free in preference to *N. coenophialum*-infected tall fescue pseudostem sections. Pseudostems are arranged to alternate infected (e.g., upper left) and endophyte-free.

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This work was supported by the United States Department of Agriculture, NRI grant 95-37303-1678, and by National Science Foundation grant DEB-9408018.

Publication number 96-12-208 of the Kentucky Agricultural Experiment Station published with the approval of the director.

Publication no. D-1997-1223-04F
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Samuels, & Christensen) Glenn, Bacon, & Hanlin (Table 1) (8). In tall fescue, *N. coenophialum* causes enhanced tillering and root growth, increases drought tolerance (1), and protects against certain nematodes (25), fungal pathogens (18), insect herbivores (41), and mammalian herbivores (2). In perennial ryegrass and fine fescues, endophyte protection from insects is well established (10,41). Both tall fescue-*N. coenophialum* symbiote and the perennial ryegrass-*N. lolii* symbiote accumulate compounds toxic to grazing mammals (38). Of course, all of these features are beneficial to the grass hosts. Yet it may seem enigmatic that tall fescue with *N. coenophialum* and perennial ryegrass with *N. lolii* are among the most important forage grasses on two continents (the former in the United States, the latter in Europe, Australia, and New Zealand) despite their protection from grazing livestock. The underlying reasons are the good forage and agronomic characteristics of the grasses as well as their exceptional fitness and low management requirements when infected by the endophytes.

In turfgrasses, endophytes can clearly add value by providing biological protection of the plants, while livestock toxicosis is not considered a problem. Many turf-type tall fescue cultivars released between 1980 (the year that Rebel was released) and the early 1990s had only low to moderate levels of endophyte infection (15). More recently, awareness of endophyte benefits has grown, so that turf breeders selecting tall fescue either use infected source material or inoculate with a selected strain of the endophyte. In perennial ryegrass, the use of endophyte-infected turf-type cultivars was earlier and more systematic than in tall fescue. Many dozens of "endophyte-enhanced" (i.e., symbiotic) cultivars have been released during the past decade (15). Symbiosis of fine fescue species with *Epichloë festucae* Leuchtman, Schardl, & Siegel also confers important benefits (43).

Life Cycles

There are two methods of transmission for *Epichloë* or *Neotyphodium* species (Figs. 2 and 3). Many (including all asexual species) can systemically infect seeds produced by the infected mother plant (vertical transmission) (Fig. 3B). Sexual species can be transmitted from an infected plant to developing seeds of a neighboring, uninfected plant (contagious or horizontal transmission). Some *Epichloë* species can transmit by both pathways. Choke disease (Fig. 3C and D) is the developmental stage required for the sexual cycle and contagious spread of *Epichloë* species. Choke disease affects only systemically infected plants and is the only macroscopic sign of the infection. Before inflorescences can emerge from the boot, the fungus prolifer-

ates to form an obvious "choke" (a conidiogenous stroma) that engulfs the inflorescence and the flag leaf sheath that surrounds it (Fig. 3C). The affected inflorescence develops no further. Choke disease is the only stage in which there are external signs of the fungus and in which host plants exhibit symptoms of infection. All *Epichloë* species investigated to date are heterothallic, and there are two opposite mating types (29,58). A fly (*Phorbia* sp.) feeds and lays eggs on chokes, and also transfers spermatia between chokes to mate the fungus (4). After a few weeks, the sexual *Epichloë* spores (ascospores) are forcibly ejected from the stroma (now a mature ascus). An ascospore that happens to land on a grass floret may initiate fungal growth down the stigma to the ovule. Indeed, we recently demonstrated that contagious spread of *E. typhina* is initiated by the sexual spores and results in infected seeds (7).

The asexual *Neotyphodium* species probably disseminate only vertically and never contagiously. In a recent report not-

ing emergence of sparse sporogenous hyphae from endophyte-infected wild grasses, White et al. (59) suggested revisiting the issue of contagious spread. However, successful inoculation of seedlings (27) is accomplished only with scrupulous attention to seedling age, site of wounding, and carefully controlled (and artificial) growth conditions after inoculation. Unwounded seedlings are not infected (27), and infection of adult plants has only been accomplished under unusual and artificial circumstances (C. L. Schardl and M. R. Siegel, unpublished). Furthermore, the genetic uniformity of endophytes from tall fescue in North America (28) indicates that infections by *Epichloë* spores have occurred rarely or not at all since the grass was introduced here. However, superinfections (simultaneous infections) by *Epichloë* and *Neotyphodium* are likely responsible for the complex history of interspecific hybridization that characterized the evolution of tall fescue endophytes in Europe and North Africa (53). A similar history is indicated for a rare endophyte genotype

Table 1. Some symbioses of forage grasses with *Epichloë* and *Neotyphodium* endophytes and their symbiosis-related alkaloids^a

Species	Hosts	EA	ID	SAP	PP	Endophyte transmission ^b
<i>Neotyphodium coenophialum</i>	<i>Festuca arundinacea</i> var. <i>genuina</i>	+/-	-	+	+	
<i>N. lolii</i>	<i>Lolium perenne</i>	+/-	+/-	-	+/-	
<i>N. uncinatum</i>	<i>F. pratensis</i>	-	-	+	-	
<i>Epichloë festucae</i>	<i>Festuca</i> spp.	+/-	+/-	+/-	+/-	
<i>E. typhina</i>	Various C3 grasses	-	-	-	+/-	—

^a Abbreviations: EA, ergot alkaloids; ID, indole diterpenes (tremorgens, such as lolitrem B); SAP, saturated aminopyrrolizidines (lolines); PP, pyrrolopyrazines (peramine); +, found in all associations to date; +/-, found in some but not all associations; -, found in none of the associations to date. Alkaloid data are from references 5 and 49.

^b | = vertically transmitted by systemic infection of seeds; — = horizontally transmitted; |— = capable of both vertical and horizontal transmission.

Fig. 2. Coordinated life cycles of *Epichloë festucae* (mating population II) and its host grass, *Festuca rubra*. From Schardl et al. (46).

Fig. 3. Stages in life cycles of *Epichloë* species and their asexual relatives. (A) *Neotyphodium coenophialum* growing intercellularly in the leaf sheath of its host grass, tall fescue. (B) *N. coenophialum* in the aleurone layer of a tall fescue seed. In A and B, fungal hyphae were stained with aniline blue. (C) Flowering tillers of an *Elymus virginicus* plant systemically infected with *Epichloë* sp. Healthy inflorescences have emerged from the tillers at the center and right, but the tiller at left exhibits the choke disease symptom of the fungus. The symptomatic inflorescence will not emerge, but the asymptomatic inflorescences will produce healthy seeds infected with the symbiont. (D) Perithecia of *Epichloë typhina* embedded in the stroma surrounding the flag leaf sheath of the host plant (in this case, perennial ryegrass).

from perennial ryegrass, although most endophytes of this grass are similar to *N. lolii* and closely related to *E. festucae* (47).

Evolution of Sexual *Epichloë* Species

Evolution of *Epichloë* species and their asexual relatives—*Neotyphodium* species—follows some surprising pathways. The first genetic documentations of inter-specific hybrid fungi were the molecular systematic analyses of tall fescue endophytes (53) and perennial ryegrass endophytes (47). In order to conclude that inter-specific hybridization has occurred, the DNA sequence relationships of nonhybrid species had to be discerned. This required that the true biological species relationships among sexual *Epichloë* species had to be established by mating tests: no mean task for these fungi (29). Three underlying evolutionary processes characterize these fungi: genetic isolation of biological species (mating populations), cospeciation with grass hosts, and interspecific hybridization.

Our best information to date indicates there are nine mating populations of *Epichloë* species. In keeping with a trend toward describing morphospecies that correspond to interbreeding populations (the biological species concept of Ernst Mayr [32]), four new *Epichloë* species have been described. *Epichloë amarillans* J.F. White and *E. baconii* J.F. White (56,57) are probably equivalent to mating populations IV and V, respectively (46). These two species are associated with *Agrostis*, *Calamagrostis*, and *Sphenopholis* species (all in tribe *Aveneae*), but mating population IV is North American and mating population V is Eurasian. Another Eurasian species, *E. festucae*, is synonymous with mating population II (29) and is associated with numerous fescues (*Festuca* species). Undescribed *Epichloë* species include mating populations III, VIII, and IX from North America and mating populations VI and VII from Eurasia. Mating population III is associated with *Elymus* and *Hystrix* species, VI with *Bromus* species, VII with *Brachypodium sylvaticum* (Huds.) Beauv., VIII with *Glyceria striata* (Lam.) Hitchc., and IX with *Brachyelytrum erectum* (Schreb.) Beauv. Mating population I is the most complex and problematic. This Eurasian mating population encompasses strains with diverse DNA sequences, morphologies, and host specificities (46).

Mating population I includes two morphospecies, *E. clarkii* J.F. White and *E. typhina* sensu stricto (46). The morphological distinction between these species is that ascospores (meiotic spores) of the former disarticulate at a medial septum before ejection from the perithecia, whereas *E. typhina* ascospores remain whole (56). In an expanded survey of these two species, the distinction between them

became ambiguous because most mating tests indicate they are interfertile (46). However, morphospecies *E. clarkii* is restricted to the grass host *Holcus lanatus* L., whereas *E. typhina* has been isolated from *Anthoxanthum odoratum* L., *Brachypodium pinnatum* (L.) Beauv., orchardgrass (*Dactylis glomerata* L.), perennial ryegrass, timothy (*Phleum pratense* L.), and bluegrasses (*Poa* species). DNA sequence data and isozyme profiles suggest that *E. clarkii* is genetically isolated from *E. typhina* in nature despite its interfertility with *E. typhina* in test matings. There is no indication of genetic isolation of *E. typhina* populations in orchardgrass, timothy, or the bluegrasses; but it is possible that those in *B. pinnatum*, *A. odoratum*, and perennial ryegrass represent genetically isolated populations. It is unknown what keeps *E. clarkii* and these other groups isolated from each other and the rest of the mating population I genotypes, but specificity for host species and for races or species of the symbiotic *Phorbia* fly are obvious possibilities. *Epichloë* species are dependent on

host grasses for a growth niche and on the flies for fertilization in the sexual life cycle that allows their contagious spread (7).

An unexpected connection emerged between *E. typhina* (mating population I) and the *B. sylvaticum* symbionts (mating population VII) (46). DNA sequences show that the speciation of these mating populations did not follow the cladistic model normally assumed in evolutionary biology. In cladistic speciation, one ancestral species is replaced by two genetically isolated daughter species. In contrast, mating population VII appears to have arisen within *E. typhina* (Fig. 4), which otherwise maintained its identity as a species. Thus, *E. typhina* is paraphyletic to the *B. sylvaticum* symbionts, such that mating populations I and VII form a clade (i.e., a single branch of the evolutionary tree). There is a particularly close relationship between isolates from host genus *Brachypodium*. The *tub2* phylogeny places mating population VII very close to an *E. typhina* strain from *B. pinnatum* (Fig. 4). This might be expected based on the

Fig. 4. Phylogenetic relationships of β -tubulin gene sequences of *Epichloë* species and asexual endophytes. Each of the *Epichloë* species isolates had a single gene, and the position is indicated by roman numerals I to IX, indicating mating populations (i.e., biological species). The β -tubulin genes of asexual endophytes are indicated by shapes as follows: black triangle, the single-copy gene in *Neotyphodium lolii*; open triangles, the two copies in the hybrid endophyte (designated LpTG-2) of perennial ryegrass; black square, the single copy in *N. uncinatum*; open squares, the three copies in *N. coenophialum*; and open pentagons, the two copies in a hybrid endophyte of *Poa ampla*.

congeneric relationship of the hosts, but the isolate from *B. pinnatum* was interfertile with mating population I and not with VII. Furthermore, this isolate is like other mating population I strains in that it completely chokes its host and is not vertically transmitted. In contrast, the *B. sylvaticum* symbionts are vertically transmitted in seeds, are interfertile with each other, and are not interfertile with the *B. pinnatum* symbiont or other mating population I strains. Interestingly, based on *tub2* and rDNA sequences, the root of the *E. typhina*–*E. clarkii*–mating population VII clade is very close to the root of the entire genus. Mating populations I and VII may represent the “living fossils” of genus *Epichloë*.

Another unexpected relationship involves *E. typhina* and mating population VIII (46). Again, mating population I is Eurasian, but VIII has been isolated only from the North American grass *G. striata*. However, very similar rDNA sequences were identified in a mating population VIII isolate and several *E. typhina* isolates. Also, the mitochondrial plasmid *Callan* is found only in mating populations I and VIII (6). The connection between these mating populations is not corroborated by *tub2* sequence relationships (Fig. 4). In fact, the incongruity between rDNA and *tub2* phylogenies suggests that mating population VIII is an interspecific hybrid (46).

Fig. 5. Comparative gene phylogenies of C3 grass tribes (left) and *Epichloë* species or mating populations (right). The grass phylogeny is based on the chloroplast DNA data of Davis and Soreng (11), and the *Epichloë* phylogeny is based on β -tubulin gene sequences (46). *Epichloë* mating populations II through IX are shown opposite the host tribes with which they appear to have cospeciated. An arrow indicates an apparent host species transfer (host jump) in the evolution of mating population VIII, which evidence from the rRNA genes suggests had a hybrid origin. Not shown is mating population I (*E. typhina* and *E. clarkii*), which cannot have cospeciated because it lacks host specificity.

Grass–*Epichloë* Cospeciation

Cospeciation, as an important trend in *Epichloë* evolution, is suggested by molecular phylogenies of these fungi (46) and their host grasses (11) (Fig. 5). Such cospeciation must be diffuse, because *Epichloë* species do not necessarily specialize to individual grass species but are almost always restricted to related grass genera within a tribe. Relationships between tribes of cool-season grasses have been well elucidated by tracking mutations that create or eliminate restriction endonuclease cleavage sites in the chloroplast genomes (11). There is a close relationship between four large tribes that include many known hosts of *Epichloë* species. These four tribes—which we will consider a “crown group”—are: (i) *Poeae*, which includes host genera *Dactylis*, *Holcus*, *Festuca*, *Lolium*, and *Poa*; (ii) *Aveneae*, which includes *Agrostis*, *Anthoxanthum*, *Calamagrostis*, *Phleum*, and *Sphenopholis*; (iii) *Triticeae*, which includes *Elymus* and *Hystrix*; and (iv) *Bromeae*, which includes *Bromus*. Within this group, *Poeae* and *Aveneae* are sister tribes, and *Triticeae* and *Bromeae* are sister tribes. More deeply rooted host tribes are: (v) *Meliceae*, which includes *Glyceria*; (vi) *Brachypodieae*, which includes *Brachypodium*; and (vii) *Brachyelytreae*, with its sole species *B. erectum*. An eighth tribe, *Stipeae* (*Stipa* spp.) has numerous asexual endophytes (23) but is not known to host sexual *Epichloë* species.

The crown group of tribes hosts a crown group of *Epichloë* species indicated by *tub2* gene phylogeny (Fig. 5). Three species associated with sister tribes *Poeae* and *Aveneae* are *E. festucae*, *E. amarillans*, and *E. baconii* (mating populations II, IV, and V, respectively). Two sister species—mating populations III and VI—are symbiotic with sister tribes *Triticeae* and *Bromeae*, respectively. Midpoint rooting of the *tub2* phylogeny indicates that three deeply rooted species—mating populations VII and IX—are associated with the deeply rooted host tribes *Brachypodieae* and *Brachyelytreae*, respectively. Phylogenetic relationships of rDNA sequences corroborate the *tub2* phylogenies for these seven *Epichloë* mating populations. Thus, the phylogeny of seven *Epichloë* biological species mirrors the phylogeny of their hosts.

In contrast to the other *Epichloë* species, cospeciation is not indicated for mating populations I and VIII. These two mating populations give unexpected gene phylogenies suggestive of unusual evolutionary origins. In the case of mating population VIII, a history of cospeciation may have become obscured by interspecific hybridization (46). The rDNA phylogeny suggests cospeciation, but its *tub2* phylogeny is not consistent with this hypothesis. Mating population I could not have cospeciated because it has not specialized to a host

species, genus, or tribe (Fig. 5). Instead, its hosts are in sister tribes *Poeae* and *Aveneae*, plus the deeply rooted tribe *Brachypodieae*. Although some individual genotypes of *E. typhina* have specialized to host genera or species (6), their specialization does not cause or correspond to the evolution of genetic barriers to mating. Thus, the diverse *E. typhina* genotypes, together with *E. clarkii*, remain in a single mating population.

The conclusion remains tentative that mating population VIII is an interspecific hybrid, and the possibility must be tested by phylogenetic reconstruction of more genes. Hybrid origins of plant species are common (60), and several examples of hybrid animal species have recently come to light (3). However, there are few examples of hybrid fungi. The best documented fungal hybrids are asexual descendants of the *Epichloë* species, discussed in the next section.

Evolution of Asexual Endophytes

The asexual *Neotyphodium* endophytes share the characteristic of many *Epichloë* species of transmitting via seeds from infected mother plants to subsequent plant generations. The asexual endophytes simply differ from their sexual relatives in lacking the sexual life cycle, and for this reason, they lack the ability to spread contagiously. However, the evolutionary relationships of asexual *Neotyphodium* and sexual *Epichloë* species are rarely simple.

The most straightforward relationship known so far between asexual and sexual endophytes is that of *N. lolii* and *E. festucae*. *N. lolii* is symbiotic with perennial ryegrass, a host with close affinity to the fescues (22,61). Sexual *E. festucae* is unknown from perennial ryegrass. Also, the presexual choke state has never been observed for *E. festucae* isolates artificially introduced into perennial ryegrass (C. L. Schardl and M. R. Siegel, unpublished). These observations suggest that perennial ryegrass may suppress the choke state. In this way, *E. festucae* that naturally infects this grass would be rendered asexual. However, it should be noted that perennial ryegrass is not fundamentally resistant to choke disease. *E. typhina* also occurs naturally in this grass, and when it does, it causes choke on all inflorescences. Also, diverse genotypes of *N. lolii* have been used as males in extensive mating tests on stromata of *E. festucae*, but all such tests failed to yield ascospores (C. L. Schardl and M. R. Siegel, unpublished), suggesting that, however *N. lolii* evolved, it is now truly asexual.

There are no known asexual endophytes that are direct descendants of *E. typhina*, although *E. typhina* has contributed by interspecific hybridization to the evolution of many (Fig. 6). A triangular relationship

exists between *E. typhina* from perennial ryegrass and two asexual endophytes of this host: *N. lolii* and a rare asexual endophyte (designated LpTG-2) from the same host (Fig. 6A). Most single-copy genes in *E. typhina* and *N. lolii* are duplicated in LpTG-2. Analysis of isozyme profiles and DNA sequences indicates that the copies of each duplicated gene in LpTG-2 come from different *Epichloë* species. The phylogenetic relationships of these genes identify *N. lolii* and *E. typhina* as the likely ancestors (9,47). Electrophoretic karyotype analysis (33; C. L. Schardl and G. A. Kuldau, unpublished) confirms that the genome of LpTG-2 is much more complex than that of *N. lolii*, *E. festucae*, or *E. typhina*. Genome size estimates (C. L. Schardl and H. F. Tsai, unpublished) indicate that LpTG-2 has nearly twice the DNA of either *E. typhina* or *E. festucae* (both estimated at 29 million base pairs). Thus, LpTG-2 is an apparent diploid or near-diploid that arose by hybridization of two species.

Tall fescue has an extraordinary diversity of symbiotic *Neotyphodium* species, all of which are interspecific hybrids (53). One of the tall fescue endophytes is *N. coenophialum*, whereas the others are loosely grouped as FaTG-2 and FaTG-3 (5). Interspecific hybrid origins were documented for two genotypes each of *N. coenophialum* and FaTG-2, and for the only known genotype of FaTG-3. At least four *Epichloë* or *Neotyphodium* species were identified as ancestral to tall fescue endophytes. The three likely ancestors of *N. coenophialum* are *Neotyphodium uncinatum* (Gams, Petrini, & Schmidt) Glenn, Bacon, & Hanlin, *E. baconii*, and *E. festucae* (Fig. 6B). Of these, *N. uncinatum* was transmitted from a maternal meadow fescue (*Festuca pratensis* Huds.) during the plant hybridization that gave rise to hexaploid tall fescue (22,61). In reproducing this first step in tall fescue evolution, we successfully transmitted *N. uncinatum* to meadow fescue hybrids with *F. arundinacea* var. *glaucescens* Boiss. (G. C. Eizenga, C. L. Schardl, and T. D. Phillips, unpublished). We postulate that two subsequent ascospore-mediated infections of this early tall fescue-*N. uncinatum* symbiotum gave rise to *N. coenophialum* (45). It is unlikely that an endophyte would have been contributed by the paternal ancestor, since male transmission of *Neotyphodium* endophytes has not been documented. However, this possibility needs to be checked by phylogenetic analysis of *F. arundinacea* var. *glaucescens* endophytes. Among the other groups of tall fescue endophytes, FaTG-2 has *E. festucae* and *E. baconii* ancestors, and FaTG-3 was derived from hybridization of *E. baconii*, or more probably of FaTG-2, with *E. typhina*.

The tall fescue-*N. coenophialum* symbiotum is among the most complex of unified genetic entities in the biosphere. This

symbiotum has a hybrid plant and hybrid fungal nucleus, plus genomes of the plant mitochondrion, plant chloroplast, and fungal mitochondrion. All but the plant nucleus are transmitted clonally in the maternal grass lineage.

The origin of *N. uncinatum* remains to be fully elucidated. The *tub2* phylogeny (Fig. 4) indicates membership in the clade with mating populations I and VII, and a particularly close relationship with an *E. typhina* isolate from *Poa pratensis* L. (Kentucky bluegrass). The rDNA sequence from *N. uncinatum* does not group with those of any extant *Epichloë* species, suggesting *N. uncinatum* in an ancient hybrid (C. L. Schardl, unpublished).

Alkaloids and Livestock Toxicosis

Some, but not all, of the protective nature of the endophytes is due to antiinsect and antimammalian alkaloids. Four alkaloid classes have so far been associated with the fungal endophytes: the ergot alkaloids (e.g., lysergic acid and ergovaline), the indole diterpenes (e.g., paxilline and lolitrem B), a pyrrolopyrazine (peramine), and the saturated aminopyrrolizidines (loline, norloline, *N*-acetyl loline, and *N*-formyl loline, etc., collectively called "lolines"). Three of the four alkaloid classes (the exception being lolines) have been produced by endophytes grown in culture (24,30,35,37,40,51; K.-R. Chung, F. F. Fannin, L. P. Bush, and C. L. Schardl, unpublished), although production in planta is much more consistent. All four classes have antiinsect activities (37,40), and activities against mammals have been reported for all but peramine (13,38). The most potent against insects are peramine and lolines (10), whereas those most potent against grazing mammals are indole diterpenes and ergot alkaloids (38). Lolitrem B is associated with the malady "ryegrass staggers," most commonly observed in sheep grazing perennial ryegrass in New Zealand. Ergovaline is associated with various maladies often observed among cattle that graze *N. coenophialum*-infected tall fescue, and collectively called "(tall) fescue toxicosis." Symptoms of tall fescue toxicosis heavily depend on the physiological state of the affected animal and temperature extremes (14,38). For example, when the toxin is coupled with heat stress, the animals lose control of body temperature and water balance, eat less, and exhibit fat necrosis. In cold conditions, the animals may suffer gangrene of limbs, probably because the potent vasoconstrictive activity of ergovaline reduces blood flow to the extremities. Ergovaline also reduces lactation and fertility, and especially in horses, causes fetal abnormalities, miscarriages, and stillbirths.

Epichloë and *Neotyphodium* species grow endophytically throughout the plant,

but in vegetative tissues, the greatest concentration of fungal hyphae is in and near the crown meristems. As a consequence, the greatest protection from herbivory is provided to the growing points. Furthermore, Hill et al. (19) found that endophyte-infected tall fescue plants had crowns at a greater depth (7 to 11 mm) than did uninfected plants, and that endophyte-infected plants had greater crown and tiller masses. Read and Camp (39) showed that endophyte-infected pastures had greater regrowth after grazing compared with low-endophyte pastures. These effects on growth should help endophyte-infected plants better resist grazing and overgrazing than noninfected plants. The crown is also protected from insect herbivores such as larvae of the Argentine stem weevil (*Listronotus bonariensis* Kuschel), a major

Fig. 6. Evolutionary networks depicting postulated origins of sexual *Epichloë* species and hybrid asexual endophytes. Species are designated with Roman numerals and shapes as in Figure 4. (A) The origin of the perennial ryegrass endophyte designated LpTG-2. (B) The origin of *Neotyphodium coenophialum*.

pest in New Zealand (41). The fact that endophyte tends to be sparse in leaf blades may ameliorate livestock toxicosis in pastures that are not heavily grazed. Still, when grazing animals are under heat or cold stress, toxicosis can be a severe problem (14,38).

Seeds with hyphae throughout the aleurone layer and less abundantly in the embryos (36) are also protected by the endophytes. It is very important that livestock not graze seed heads and that tall fescue be cut for hay before it begins to set seed. Ironically, although endophyte growth in seed heads is an important problem, ergot alkaloid poisoning can result regardless of whether the grass is infected with an endophyte. This is because florets of tall fescue and other open-pollinated grasses are often infected with the ergot fungus (*Claviceps purpurea* (Fr.) Tul.), a relative of the endophytes that differs in two important ways: First, *C. purpurea* is contagiously spread from its overwintering structures (ergots) in the soil and from floret to floret. Second, ergots are extremely high in ergot alkaloids (26,38). Thus, a balance of grazing pressure should be maintained to keep the pasture vegetative but not over-grazed.

The approaches for alleviating or eliminating toxicosis due to endophyte infections of the grasses are varied. The earliest approach, production and replanting with endophyte-free cultivars, has met with limited success. Opinions vary on why this approach is not widespread. It is difficult to replace 17 million hectares of endophyte-infected tall fescue in the United States, much of which is on hilly, marginal lands not intensively managed. Lower stress tolerance of endophyte-free cultivars

makes management important. Seedlings of uninfected cultivars are more susceptible to *Rhizoctonia* species (18), so they may be difficult to establish where the pathogen is present. Also, as stated above, endophyte-free plants may be less tolerant of grazing pressure. Meanwhile, more attention has steadily been directed to other possible remedies, such as alleviating toxic symptoms in livestock by thiamine supplements (14) or vaccination against ergot alkaloids (20), identifying endophyte genotypes (5,49) or grass genotypes (42) with low toxic alkaloid accumulation, or genetic engineering of the endophytes (33,54).

Genetic Engineering and Research on Livestock Toxicosis

Perhaps the disadvantages of endophyte-free tall fescue can be mitigated by well-conceived and intensive breeding for better establishment, increased drought tolerance, and greater resistance to insects, nematodes, and pathogens. A plant breeding strategy has been suggested in which endophytes are first removed from breeding populations to effectively select for (unmask) nonendophytic sources of pest resistance and stress tolerance (15). Endophyte removal can be accomplished by fungicide treatment of plants or heat treatment of seeds (12,50). The appropriate endophyte(s) might then be added, for example by inoculating seedlings (27), to enhance performance when desired. This approach has merit, but a concern is that grass-endophyte compatibility may be reduced by genetic drift or inadvertent selection (e.g., a pleiotropic effect of genetic resistance to diseases). Also, the number of plants to subsequently reinfest with the desired endophyte(s) needs to be great enough to prevent genetic bottlenecks or drift that could affect the quality of the cultivar. Finally, the effect of the reintroduced endophyte on plant physiology and selected traits may be complex and difficult to predict. Therefore, it seems prudent also to breed forage cultivars containing endophyte. The breeding lines may behave more predictably when their endophytes are replaced with new genotypes such as those genetically altered for low production of antimammalian alkaloids.

As with the genetic variation in plant germ plasms, genetic variation of endophytes is potentially of great utility. Not all endophytes are associated with livestock toxicosis. In Texas, annual ryegrass forage yields were enhanced when the plants were infected with a *Neotyphodium* endophyte, but no adverse effects on animals consuming the infected plants were reported. Forage yield was approximately 10% greater in populations infected with the endophyte than in a genetically similar population without the endophyte (34). We

are unaware of any livestock toxicosis attributable to *N. uncinatum*, the common endophyte of meadow fescue. Very high levels of lolines accumulate in meadow fescue-*N. uncinatum* symbiota, in which the antimammalian ergot alkaloids and indole diterpenes have not been detected. Endophytes from native populations of tall fescue and perennial ryegrass also vary considerably in alkaloid profiles (5) and are potentially useful for forage cultivars if they provide benefits comparable to those of the endophytes now common in commercial cultivars.

Genetic transformation technologies applied to endophytes of tall fescue and perennial ryegrass (33,54) may soon be used to specifically knock out production of alkaloids that cause livestock toxicosis. Endophytes altered in this way should still provide many of the benefits of the wild-type endophytes. Biosynthetic pathways for ergot alkaloids and indole diterpenes are complex (31,35), but genes for key steps can be targeted to eliminate these alkaloids. Probably the first to succumb will be the ergot alkaloids. There is much information on biosynthesis of ergot alkaloids by the ergot fungus, *C. purpurea* (48), a relative of *Epichloë* and *Neotyphodium* species. The first (determinant) step is synthesis of 4- γ , γ -dimethylallyltryptophan from two common cellular constituents. The enzyme for this step has been purified (16), and the gene (*dmaW*) has been cloned and characterized (55). A putative homolog of *dmaW* has been cloned from *N. coenophialum* (C. L. Schardl, unpublished) in preparation for molecular genetic studies and manipulations. Ultimately, the goal is to eliminate expression of *dmaW* in endophytes of tall fescue and other forage grasses using one or more of the gene disruption techniques that have been developed for related fungi (52).

The techniques of transformation with exogenous DNA, adapted for endophytes (54), can also be used for expression of novel genes in grass-endophyte symbiota. The concept has been tested by transformation with the histological marker, β -glucuronidase. The marker is expressed by transgenic endophyte, both in culture (Fig. 7) and in planta (33). This constitutes a successful test of concept that may be applied in the future to expression of additional protective genes, such as *Bacillus thuringiensis* δ -toxins, for protection from herbivorous insects (21).

Another potentially useful advance is the breeding of *Epichloë* endophytes. Identification of interfertile strains (29) allows these grass symbionts to be analyzed in a classical (Mendelian) genetic manner. In the process, genes controlling expression of alkaloids can be identified. For example, matings and backcrosses between strains of *E. typhina* indicate a single locus controlling peramine expression (6). Similar genetic analyses are underway with *E. festu-*

Fig. 7. Expression of β -glucuronidase by *Neotyphodium coenophialum* transformed with the *uidA* gene of *Escherichia coli*, driven by a fungal promoter. The dark blue color in six of the colonies is a histological indicator of the enzyme. The colony at bottom is the negative control, *N. coenophialum* transformed with a gene for hygromycin resistance but not *uidA*.

cae. Advantages to *E. festucae* are that it is seed-transmissible, tends to cause very little choke disease, has demonstrated beneficial effects on infected hosts (43), shows variability in alkaloid profiles among strains (49), and is related to some of the ancestors of tall fescue and perennial ryegrass endophytes (47,53). These characteristics indicate a potential for *E. festucae* genotypes to be bred for specific alkaloid profiles and uses in forage and turfgrasses.

At present, the main objective of eliminating genes for alkaloid production is to test their roles in livestock toxicosis and host fitness. Only if resulting endophytes are clearly shown to have low toxicity to livestock and wildlife, and new symbiotic cultivars are agronomically desirable, readily established, and persistent, should they be applied as a solution to the livestock toxicosis problem.

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In the Feature, "Protective grass endophytes: Where are they from and where are they going?" by Christopher L. Schardl and Timothy D. Phillips on pages 430 to 438, the caption for Figure 1C should read "Aphids (*Rhopalosiphum padi*) feeding preferentially on endophyte-free in preference to *N. coenophialum*-infected tall fescue pseudostem sections. Pseudostems are arranged to alternate infected (e.g., upper right) and endophyte-free."