Contribution of Fungal Loline Alkaloids to Protection from Aphids in a Grass-Endophyte Mutualism

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Fungal endophytes provide grasses with enhanced protection from herbivory, drought, and pathogens. The loline alkaloids (saturated 1-aminopyrrolizidines with an oxygen bridge) are fungal metabolites often present in grasses with fungal endophytes of the genera *Epichloë* or *Neotyphodium*. We conducted a Mendelian genetic analysis to test for activity of lolines produced in plants against aphids feeding on those plants. Though most loline-producing endophytes are asexual, we found that a recently described sexual endophyte, *Epichloë festucae*, had heritable variation for loline alkaloid expression (Lol⁺) or non-expression (Lol⁻). By analyzing segregation of these phenotypes and of linked DNA polymorphisms in crosses, we identified a single genetic locus controlling loline alkaloid expression in those *E. festucae* parents. We then tested segregating Lol⁺ and Lol⁻ full-sibling fungal progeny for their ability to protect host plants from two aphid species, and observed that alkaloid expression cosegregated with activity against these insects. The in planta loline alkaloid levels correlated with levels of anti-aphid activity. These results suggested a key role of the loline alkaloids in protection of host plants from certain aphids, and represent, to our knowledge, the first Mendelian analysis demonstrating how a fungal factor contributes protection to plant-fungus mutualism.

Additional keywords: amplified fragment length polymorphism (AFLP), *Festuca*, *Lolium* spp., *Rhopalosiphum padi*, *Schizaphis graminum*.

In symbiotic mutualisms partners exchange “goods and services” (Janzen 1985) and, as a result, both host and symbiont obtain a net benefit from the interaction. In many plant-microbe mutualisms, such as mycorrhizae and legumes with rhizobia, the symbiont provides a nutritional service by making available to the host plant-limiting nutrients such as phosphorus or fixed nitrogen. Another widespread type of mutualism is the association of grasses with seedborne fungal symbionts (endo-

phytes), in which the principal service provided by the fungus is protection from biotic and abiotic environmental stresses. The best studied of these symbionts, *Epichloë* and *Neotyphodium* spp. (sexual and asexual endophytes, respectively, in the family Clavicipitaceae), protect host plants from herbivores, parasites, competition, and drought (Clay 1990; Schardl and Clay 1997). In these protective mutualisms, the symbionts may produce any of several classes of alkaloids, including lolines (saturated 1-aminopyrrolizidines with an oxygen bridge) (Fig. 1), peramine (a pyrrolopyrazine), ergot alkaloids, and indole diterpenes (Bush et al. 1997).

Lolines are rare alkaloids that occur almost exclusively in many grasses associated with *Epichloë* and *Neotyphodium* spp. Pure lolines are toxic when fed or applied to insects (Bush et al. 1993; Riedell et al. 1991). Furthermore, Siegel et al. (1990) report that when *Rhopalosiphum padi* (bird cherry-oat aphids) are fed natural grass-endophyte combinations (symbiota) with lolines they have significantly reduced survival, compared with aphids on symbiota with no detectable lolines. Taken together, such observations suggest a protective role for lolines, but are not definitive because the toxicity assays were necessarily artificial, while the tests on plants involved comparisons among different grass species and were confounded by variation in profiles of other fungal and plant metabolites. It is conceivable, for example, that lolines and antagonism to *R. padi* are simply two correlates of endophyte relationships. A Mendelian genetic analysis would provide a more definitive test of alkaloid roles, but until recently there were no loline-producing endophytes with a known sexual state.

Epichloë festucae, a recently described fungal symbiont of *Festuca* and *Lolium* spp. grasses (Leuchtman et al. 1994), has the appropriate characteristics as a genetic model to test ecological roles hypothesized for fungal factors such as loline alkaloids. This fungus is a close sexual relative of the asexual *Neotyphodium* species for which alkaloid profiles and anti-insect activities are best characterized. Plants with symbiotic *E. festucae* are reported to have enhanced resistance to insects (Funk et al. 1994), and natural *E. festucae*-grass symbiota vary widely in alkaloid profiles (Bush et al. 1997). Typically, giant fescue (*Lolium giganteum* = *Festuca gigantea*) is symbiotic with a loline alkaloid producing *E. festucae* genotype (Leuchtman and Schardl 1998; Leuchtman et al. 2000; Siegel et al. 1990). Interestingly, lolines are also produced by the asexual *Neotyphodium* species that are usually present in several close relatives of this grass: tall fescue (*Lolium arundinaceum* = *Festuca arundinacea*), meadow fescue (*Lolium pratense* =

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Festuca pratensis), and numerous annual ryegrass species (Siegel et al. 1990; TePaske et al. 1993). All of these grass species are widespread in their native European habitats as well as other continents where they have been naturalized, and both native and naturalized populations have high frequencies (often over 90%) of endophyte-symbiotic individuals (Pfannmüller et al. 1997; Zabalgoitia et al. 1997). Therefore, an analysis of the possible anti-insect role of lolines is relevant to the ecology of these important grasses.

Here we report a Mendelian test for genetic linkage of anti-aphid activity with expression of lolines, followed by a dose-response analysis to further test the role of lolines. We identified and crossed sexually compatible *E. festucae* isolates that qualitatively differed in loline alkaloid expression. Segregation ratios and linkage to DNA polymorphisms were consistent with control of loline alkaloid expression (Lol^+) and nonexpression (Lol^-) phenotypes by allelic variation at a single locus. We then used the grass-endophyte associations generated in the genetic analysis to compare effects on two aphid species of full-sibling fungal progeny segregating for Lol^+ and Lol^- phenotypes. We predicted that if lolines are responsible for anti-aphid effects in grass-*E. festucae* associations, then the Lol^+ phenotypic class should reduce aphid survival relative to the Lol^- class. To test whether the anti-aphid activity of the Lol^+ progeny was likely due to lolines rather than to a different, genetically linked trait, we investigated whether quantitative variation in the alkaloid levels correlated with the aphid response. The significance of loline alkaloid effects on aphids, and of this genetic approach to study factors mediating these mutualisms, is discussed.

RESULTS

Natural giant fescue-*E. festucae* symbiota contain lolines, whereas none have been detected in natural red fescue-*E. festucae* symbiota (Leuchtmann et al. 2000; Siegel et al. 1990). To test whether fungal genotype determined loline alkaloid production, isolate 189 from red fescue (*Festuca rubra* subsp. *rubra*) and isolate 434 from giant fescue were each introduced into eight plants of meadow fescue (a close relative of giant

fescue) (Darbyshire 1993). Plants with isolate 434 had total loline alkaloid levels ranging from 161 to 388 ppm, whereas plants with isolate 189 never exhibited even trace lolines (threshold of detection, 10 ppm). These two isolates were then mated, and a series of backcrosses and sibling crosses were conducted to assess the heritability of loline alkaloid expression (Table 1). In all crosses, the $Lol^+ : Lol^-$ segregation ratio was not significantly different from 1:1. There was no heterogeneity between crosses, and the same segregation pattern was obtained regardless of whether the maternal parent was Lol^+ or Lol^- . Since *E. festucae* is haploid, this result was consistent with the hypothesis that the phenotypic difference between the two original parent isolates was controlled by allelic differences at a single nuclear locus, hereafter designated *LOL*. We obtained additional support for this hypothesis by identifying two DNA polymorphisms apparently linked to *LOL* (Fig. 2, bands A and B). These polymorphisms were identified as amplified fragment length polymorphism (AFLP) bands that segregated in a Mendelian fashion among the 56 BC_1 progeny analyzed from the 189 $Lol^- \times$ 1035.30 Lol^+ cross. The band A polymorphism was identified in 24 of the 28 Lol^+ progeny and two of the 28 Lol^- progeny, while band B strictly cosegregated with the Lol^+ phenotype. Thus, Mendelian tests and linkage analysis of DNA polymorphisms indicated that loline alkaloid expression was highly heritable and suggested simple genetic control.

We took advantage of this Mendelian inheritance to further investigate the hypothesis that lolines can protect against certain aphid species (Fig. 3). We tested the effects of plants symbiotic with segregating Lol^+ or Lol^- BC_1 progeny as well as symbiont-free meadow fescue (E^-) on *Schizaphis graminum* (greenbug) and *R. padi*. For both aphid species the overall effect of treatment (Lol^+ , Lol^- , or E^-) on the number of live aphids was highly significant (analysis of variance [ANOVA]: *R. padi* $F_{2,39} = 60.073$, $P < 0.001$; *S. graminum* $F_{2,39} = 79.467$, $P < 0.001$). For both aphid species, there was dramatically decreased survival and/or reproduction when fed meadow fescue symbiotic with Lol^+ BC_1 progeny, but not with the sibling Lol^- progeny (*R. padi* $P < 0.001$; *S. graminum* $P < 0.001$). Also, the number of living aphids for plants symbiotic with Lol^- progeny was not significantly different from the number for symbiont-free meadow fescue (*R. padi* $P = 1.000$; *S. graminum* $P = 0.194$). Thus, the effect of Lol^+ progeny against the aphids was mostly or entirely attributable to loline alkaloids or characteristics genetically linked to *LOL*.

A dose response of aphids to loline alkaloid levels further supported a protective role for these alkaloids. In the aforementioned tests when loline alkaloid levels were 67 to 576 ppm (mean $\pm$ SE = 228 ± 40) the Lol^+ progeny caused nearly complete killing of the aphids. However, in a separate experiment on *S. graminum* (Fig. 4), the loline alkaloid levels in many of the plants were lower (range 30 to 192 ppm; mean $\pm$ SE = 83 ± 13). (Reasons for the lower alkaloid levels are unknown, but possibilities include different physiological ages of the plants and environmental variations in the greenhouse.) Even with lower average alkaloid levels there was a highly significant effect of treatment (Lol^+ [$n = 14$], Lol^- [$n = 10$], or E^- [$n = 20$]) on the number of live aphids (ANOVA: $F_{2,41} = 6.22$, $P = 0.004$), the difference in numbers of live aphids for associations with Lol^+ versus Lol^- progeny was highly significant ($P = 0.004$), and the effect of plants with Lol^- progeny

Fig. 1. Minimum energy structures of the two most abundant saturated 1-aminopyrrolizidines (lolines) in natural giant fescue symbiosis with *Epichloë festucae*. Nitrogen (N) and oxygen (O) atoms are indicated, carbon atoms are unlabeled, and hydrogen atoms are not shown.

was not significantly different from that of E⁻ plants ($P = 0.26$). When number of surviving aphids was regressed against logarithm of loline alkaloid concentration (as is common for dose response analysis), there was a significant negative correlation (Pearson correlation $r = -0.55$, $P = 0.04$).

Additional *S. graminum* assays were conducted with meadow fescue ecotypes possessing *N. uncinatum*, the most common endophyte of meadow fescue. Among these symbiots ($n = 21$), the number of live aphids (mean $\pm$ SE = 2.57 ± 0.88) and the number of dead aphids (25.10 ± 0.52) indicated almost no aphid survival or reproduction. Direct statistical comparison with either class of *E. festucae* progeny or the endophyte-free plants would be inappropriate because they involved different host genotypes. Also, since Mendelian analysis is precluded for *N. uncinatum* (an asexual fungus), and mutants are unavailable, the effect of this endophyte cannot be definitively ascribed to lolines. Nevertheless, considering the dose response to Lol⁺ *E. festucae* (Fig. 4B), this extreme aphid mor-

tality was fully in keeping with the high levels of lolines in *N. uncinatum*-associated meadow fescue (366 to 6,060 ppm; mean $\pm$ SE = 2628 ± 353).

DISCUSSION

We present evidence, based on both genetic linkage and an in vivo, dose-response study, that expression of a secondary metabolite class (lolines) by the endophyte *E. festucae* provides significant protection to host plants from the aphid species *S. graminum* and *R. padi*. Previous studies had indicated that lolines have demonstrable insecticidal activities. When sprayed on *S. graminum*, their activities are comparable to nicotine, with reported LD₅₀ values of 343 ppm for N-acetyllooline and 437 ppm for N-formyllooline (Riedell et al. 1991). Since these levels are comparable to those observed in plants with Lol⁺ *E. festucae*, direct toxicity may have contributed to the protective effect. In the tests of anti-aphid activi-

Table 1. Segregation of loline alkaloid expression phenotypes in *Epichloë festucae* crosses

Generation (family no.)	Parents ^a	N	Segregation ratio (Lol ⁺ :Lol ⁻)	Sum statistics ^b	df	G	G _{adj} ^c	P ^d
F ₁ (1035)	189 (-) $\times$ 464 (+)	49	29:20		1	1.662	1.656	0.20
F ₂	1,035.33 (+) $\times$ 1,035.01 (-)	19	9:10		1	0.053	0.051	0.82
F ₂	1,035.33 (+) $\times$ 1,035.16 (-)	29	14:15		1	0.034	0.034	0.85
BC ₁ (1071)	189 (-) $\times$ 1,035.30 (+)	88	44:44		1	0.000	0.000	1.00
BC ₁	189 (-) $\times$ 1,035.33 (+)	22	12:10		1	0.182	0.178	0.67
BC ₁	1,035.33 (+) $\times$ 189 (-)	16	8:8		1	0.000	0.000	1.00
BC ₂	189 (-) $\times$ 1,071.071 (+)	20	10:10		1	0.000	0.000	1.00
BC ₂	189 (-) $\times$ 1,071.092 (+)	21	13:8		1	1.202	1.174	0.28
				G _T	8	3.133		0.93
				G _P	1	0.742		0.39
				G _H	7	2.391		0.94

- ^a Parents are indicated as female $\times$ male, with phenotypes designated (+) for Lol⁺ and (-) for Lol⁻.
^b The pooled G-statistic (G_P) was calculated from pooled data for the two progeny classes (i.e., 139 Lol⁺:125 Lol⁻). The G statistic for heterogeneity was G_H = G_T - G_P (Sokal and Rohlf 1995). Proportions of Lol⁺ and Lol⁻ progeny were uniform across replicates (i.e., crosses) as indicated by the nonsignificant test for heterogeneity (G_H). The sum of G statistics for all eight crosses provided a G total (G_T).
^c Williams correction for continuity applied for individual crosses, in which $n < 200$ (Sokal and Rohlf 1995).
^d P values > 0.05 indicate that the segregation ratio was not significantly different from the null hypothesis of single locus control (i.e., 1:1). P values for individual crosses refer to G_{adj}.

Fig. 2. Amplified polymorphic DNAs linked to the genetic locus governing loline alkaloid expression in *Epichloë festucae*. Central lanes represent the Lol⁻ parental isolate 189, the Lol⁺ parent 464, and the 189 $\times$ 464 progeny (1035.30; Lol⁺) used in the backcross to 189. Other lanes are 189 $\times$ 1035.30 backcross progeny with the loline alkaloid expression phenotypes indicated. Including those shown here, a total of 28 Lol⁺ and 28 Lol⁻ backcross progeny were analyzed. Band B (estimated at 246 bp) strictly cosegregated with the Lol⁺ phenotype; band A (estimated at 302 bp) occurred in 24 out of 28 Lol⁺ progeny and was absent from 26 out of 28 Lol⁻ progeny. Examples of progeny with recombination between band A and LOL are indicated with an asterisk (*). For band B, Lol⁺ parents and progeny all have a double-band pattern with a more intense upper band (arrow), whereas only a faint comigrating band at that position is observed in some Lol⁻ isolates.

ties, data (not shown) for the distribution of live and dead aphids on and off the plants were suggestive of both deterrence and insecticidal activity of lolines. However, a more detailed study of aphid behavior on these plants is required to directly assess whether the aphids probed or were deterred from feeding.

To kill aphids in natural circumstances, the alkaloids must be located in the phloem sap on which these insects feed. Our observations suggest that this is the case, since lolines appear to be translocated to roots (Burhan 1984). In 6- to 14-week-old tall fescue plants symbiotic with *Neotyphodium coenophialum*, loline alkaloid levels in roots were 3 to 14% (95 to 701 ppm) of those in aboveground portions (3,430 to 4,900 ppm). It is very likely that this was due to phloem translocation of lolines, and not to root-associated endophyte. Although sparse root colonization by *N. coenophialum* has been reported (Azevedo and Welty 1995), no such root colonization has been observed in naturally growing, mature plants (Hinton and Bacon 1985). Furthermore, in Burhan's (1984) study, root colonization was undetectable by serology. Thus, the simplest explanation for the anti-aphid activity is that the aphids are killed by lolines that they ingest.

Protection of plants against aphids is significant for the ability of plants to retain their photosynthetic assimilate as well as to reduce aphid-vectored parasites. The two aphid species tested here are major vectors of *Barley yellow dwarf virus*, and symbiosis with *N. coenophialum* significantly correlates with substantially reduced incidence of this virus in tall fescue (36 versus 74% infection in the absence of *N. coenophialum*) (Mahmood et al. 1993). The tall fescue-*N. coenophialum* symbiosis consistently has >1,000 ppm of loline alkaloids and is protected from these aphids (Siegel et al. 1990). Likewise, meadow fescue with its more common endophyte, *N. uncinatum*, has high loline alkaloid levels and exhibits activity against both *R. padi* (Christensen et al. 1993) and *S. graminum* (this study).

The inheritance pattern of Lol⁺ and Lol⁻ phenotypes, together with the observed linkage of AFLPs, can be explained most simply by allelic differences at a single locus determin-

ing the different phenotypes. Under this working hypothesis, we have designated this putative locus *LOL*. The two AFLP markers provide a preliminary genetic map of *LOL* and the surrounding region, whereby the band *B* polymorphism mapped very close to or coincident with *LOL*, and the band *A* polymorphism mapped approximately 11 map units from *LOL*. The identification of band *A* as a marker with a finite, nonzero map distance from *LOL* strongly suggests that the locus is on a chromosome for which both parents have homologues; that is, *LOL* was not associated with a dispensable chromosome such as sometimes occur in fungi (Kistler and Miao 1992). Of course, the size and specific nature of *LOL* remain to be determined. Although fungal genes for a secondary (or even primary) metabolic pathway are often clustered, they can also be scattered in the genome (Keller and Hohn 1997). Thus, it could be that *LOL* contains anything from a single regulatory gene to all genes for the loline biosynthetic pathway. Also, possible genetic reasons for the Lol⁻ phenotype range from mutations in individual genes to the complete absence of genes for this pathway. For purposes of this paper, the important observation is that there is genetic control over loline alkaloid expression, thus enabling a test of linkage between an ecological effect—namely, anti-aphid activity—and these specific alkaloids.

Genetic analysis of sexual endophytes such as *E. festucae* can substantially advance our understanding of protective plant-microbe mutualisms and the roles of fungal metabolites in host protection. This paper demonstrates the advantage of *E. festucae* as a genetic model. Given its sexual cycle, known mutualistic effects, variation in alkaloid profiles, broad host range among *Festuca* and *Lolium* spp. grasses, and close relationship with other mutualistic symbionts (Schardl et al. 1997), this fungus is ideal for studies of the many fitness enhancements that have been attributed to *Epichloë* and related *Neotyphodium* species (Bush et al. 1997; Clay 1990; Schardl and Clay 1997). Ultimately, elucidating ecological roles of these symbionts will shed light on how their mutualisms have evolved, the causes of their functional diversity, consequences of their use in agriculture and land reclamation (Schardl and Phillips 1997), and their importance in natural ecosystems.

Fig. 3. Cosegregation of loline alkaloid expression and activity against the aphids *Schizaphis graminum* (left) and *Rhopalosiphum padi* (right). Shown are means $\pm$ SE. Meadow fescue plants were symbiotic with Lol⁺ ($n = 16$) or Lol⁻ ($n = 16$) BC₁ progeny, or were endophyte-free (E⁻; $n = 13$). Treatments with significant differences ($P < 0.001$) in the numbers of live aphids are indicated by different letters above the bars. To avoid confusion, analysis with dead aphid number as the dependent variable is not indicated, but aphids fed on Lol⁺ symbiots exhibited significantly greater mortality ($P < 0.001$) relative to both Lol⁻ and E⁻ treatments. Levels of total loline alkaloids in the Lol⁺ symbiots were 67 to 576 ppm.

MATERIALS AND METHODS

Chemical analysis.

Loline alkaloid extraction and gas chromatography were by a modification of Yates et al. (1990). Leaf material was harvested, freeze-dried, and powdered with a Thomas-Wiley mill fitted with a #40 screen (Arthur H. Thomas, Philadelphia, U.S.A.). For each sample, 100 mg of freeze-dried tissue was combined with 100 μ l of saturated sodium bicarbonate in a 1.5-ml microcentrifuge tube (previously heat treated to remove contaminants). The suspension was centrifuged 2 min at 15,000 $\times$ *g* to fully wet the plant material. To this was added 1,000 μ l of 5% ethanol 95% CH₂Cl₂ containing, as an internal standard, 25 mg l⁻¹ 4-phenylmorpholine. The mixture was vortexed, shaken vigorously for 30 min, and then centrifuged at 15,000 $\times$ *g* for 5 min. The organic (top) phase was transferred to a glass vial for analysis. Samples (2 μ l) were injected into a gas chromatograph equipped with a 15 m $\times$ 0.53 mm SPB-1 (dimethylpolysiloxane liquid phase) fused silica column. The gas chromatograph was equipped with a flame ioni-

zation detector. Column operating conditions were 80°C for the first 2 min, a 4°C min⁻¹ rise for 35 min, and then 220°C for 10 min. Loline alkaloid levels are given in ppm (µg g⁻¹) of dry biomass. Sums of N-formylloline and N-acetylloline levels are reported because these always constituted >95% of the aminopyrrolizidines present. Identities of the loline alkaloids in plants associated with parental and certain progeny isolates of *E. festucae* were confirmed by gas chromatography-mass spectrometry (Petroski et al. 1989): eims (70 eV). For N-formylloline m/z (rel. int.) [M-28]⁺154(25), 123(13), 110(13), 95(28), 82(100); for N-acetylloline [M]⁺196(3), 167(9), 153(12), 123(28), 95(46), 82(100).

Analysis for the presence of the pyrrolopyrazine alkaloid peramine was as previously described (Fannin et al. 1990), with purified standard and *N. coenophialum*-infected tall fescue as positive controls.

Biological materials and establishment of symbiotic associations.

Two parental *E. festucae* isolates were obtained from their natural hosts: isolate 189 was from red fescue and isolate 434 was from giant fescue (Leuchtmann and Schardl 1998). Voucher specimens have been deposited in Centraalbureau voor Schimmelcultures (CBS) as CBS 102477 and CBS 102474, respectively. Cultures were maintained on potato dextrose agar (PDA) (Difco, Detroit, MI, U.S.A.) at 21°C in the dark. Endophyte-free red fescue cv. Ensylva was supplied by R. Funk, and endophyte-free meadow fescue cv. Predix was provided by Dorothea Schmidt (Station fédérale de recherches agronomique de Changins, Nyon, Switzerland).

Parental and progeny isolates were introduced into meadow fescue (some also into red fescue) to form stable symbiota, whereby each symbiotum consisted of an individual plant with an individual isolate. The reasons for using meadow fescue were that an endophyte-free cultivar was available for this host, the grass is a close relative of giant fescue, and (in contrast to giant fescue) large numbers of plants could be maintained in the greenhouse where they survived indefinitely. The introductions were as previously described (Chung et al. 1997). Briefly, seeds were germinated and each 7-day-old seedling was inoculated by placing actively growing fungal mycelium on pin pricks near the shoot meristem. Inoculated plants were taken through a specified growth regime (Latch and Christensen 1985), and then tillers were tested by tissue-print immunoblot with antiserum specific for *Epichloë* and *Neotyphodium* species (An et al. 1993). Ramets were regularly reotted to maintain symbiota as clones in the greenhouse.

Colonies of *R. padi* and *S. graminum*, provided by S. Clement (USDA ARS, Washington State University, Pullman, U.S.A.), were maintained on wheat and barley, respectively. Aphids were transferred to 7- to 10-day-old plants every 7 to 10 days.

E. festucae matings.

Matings were conducted as described previously (Leuchtmann et al. 1994). Mature red fescue plants symbiotic with isolates of *E. festucae* were transplanted in the fall to Spindletop Farm, Fayette County, KY, U.S.A. After vernalization over the winter, fungal fruiting structures (stromata) emerged in the spring concomitantly with host flowering. The plants were then brought into the greenhouse and transplanted into 30-cm-

diameter pots with 1:3 Murray silt loam:Pro-Mix BX (Premier Brands, Red Hill, PA, U.S.A.). Spermatia, which are mitotic spores (conidia) either from stromata or fungal cultures, were transferred to stromata to initiate matings. In each mating in which stroma and spermatia were of opposite mating type, perithecia-bearing meiotic spores (ascospores) matured in approximately 4 weeks. When microscopic inspection of squashed perithecia from a stroma indicated that ascospores were mature, the stroma was placed on the lid of an overturned water agar plate. Ascospores ejected onto the agar surface began germinating and producing conidia within 48 h. To ensure purity of each progeny isolate, conidia from germinated ascospores were streaked onto PDA plates, then taken through two more rounds of single-conidium isolation. Each progeny was then reintroduced into meadow fescue seedlings as described above, and the resulting symbiota were checked for lolines. Also, the presence and identity of the symbiont in each plant were checked by reisolation from the plant followed by morphological evaluation; the morphologies of *E. festucae* conidia and colonies are readily distinguished from those of the usual meadow fescue symbiont, *N. uncinatum* (Leuchtmann 1994).

Tests for heritability of loline alkaloid expression.

Stromata of 189 (Lol⁺) were mated with spermatia of 434 (Lol⁺) to produce the first filial (F₁) generation, and the F₁ progeny were introduced into meadow fescue for alkaloid analysis. Two families of first backcross (BC₁) progeny were then generated by mating Lol⁺ F₁ progeny 1035.30 (= CBS 102475) and 1035.33 (= CBS 102476) with their Lol⁻ parent, 189. To obtain stromata for additional matings, 31 F₁ progeny were also introduced into red fescue and the resulting symbiota vernalized in the field. Only one F₁ progeny (1035.33) produced stromata, so this was the only progeny used as a female in sibling (F₂) crosses and reciprocal backcrosses. (To confirm its identity, 1035.33 was reisolated from the red fescue plants and its AFLP profile analyzed as described below.) Also, two families of second-generation backcross (BC₂) progeny were

Fig. 4. Endophyte activity against *Schizaphis graminum* in relationship to loline alkaloid levels. All Lol⁺ and Lol⁻ progeny were siblings from the 189 × 1035.30 cross (Table 1). Left, Comparison of meadow fescue plants symbiotic with Lol⁺ ($n = 14$) versus Lol⁻ ($n = 10$) full-sibling BC₁ progeny, and endophyte-free (E⁻; $n = 20$) meadow fescue. Treatments with significant differences ($P < 0.005$) in numbers of live aphids are indicated by different letters above bars. To avoid confusion, analysis with dead aphid number as dependent variable is not indicated, but aphids fed on Lol⁺ symbiota exhibited significantly greater mortality ($P < 0.001$) relative to both Lol⁻ and E⁻ treatments. Right, Negative correlation of aphid survival with the logarithm of loline alkaloid levels in the Lol⁺ symbiota. The Lol⁺ associations had total loline alkaloid levels of 30 to 192 ppm, with a mean ($\pm$ SE) of 83 ($\pm$ 13). r = Pearson correlation coefficient.

generated by mating each of two Lol⁺ BC₁ progeny (1071.071 and 1071.092) with isolate 189.

Identification and analysis of DNA polymorphisms.

AFLP DNA markers were identified by the method of Vos et al. (1995). Progeny were reisolated from symbiota of known loline alkaloid phenotype and fungal DNA was extracted by the method of Byrd et al. (1990). The DNAs were digested with restriction endonucleases *Mse*I and *Eco*RI, and the fragments ligated to adapters specific to the overhanging ends. The ligated DNAs were "preamplified" by polymerase chain reaction (PCR) with oligonucleotide primers complementary to the adapters. Then, selective primers were used for the PCR amplification step. The primers were 5'-GACTG CGTACCA-ATTCG-3' (labeled with 5'-[³²P]phosphate) for *Eco*RI-generated ends, and 5'-GATGAGTCCTGAGTAAGA-3' for *Mse*I-generated ends. The amplified DNA fragments were resolved by polyacrylamide gel electrophoresis and detected by autoradiography.

Anti-insect activity assays.

Tests of the effects of Lol⁺ and Lol⁻ BC₁ progeny on *R. padi* and *S. graminum*, and comparisons with symbiont-free plants (E⁻), involved an established assay (Siegel et al. 1990) with minor modifications. BC₁ progeny were used for comparisons of the two phenotype classes (Lol⁺ and Lol⁻). (Note that the parents in the BC₁ crosses did not express peramine, thus avoiding any confounding effect of this insect feeding deterrent, which is known to be produced by some endophytes.) Three tillers of each plant were used for each aphid test, and all remaining aboveground plant material was collected for alkaloid analysis. A thin cross section from the base of each tiller was rechecked by immunoblot for the symbiont (An et al. 1993). Twenty-five aphids at the second juvenile stage were placed into a cage with the three tillers. After 72 h at room temperature, the numbers of live and dead aphids on and off each plant were counted. However, since wingless female aphids can parthenogenically produce up to four young per day, the total number of live and dead aphids at the end of the assay often greatly exceeded the original number placed on the plant. Thus, this number actually reflected the effects on survival and/or reproduction. ANOVA was used to test for effects of phenotype (Lol⁺, Lol⁻, or E⁻) on the numbers of live aphids. We chose numbers of live aphids as the dependent variable for this analysis because it represented combined survival and reproduction of the aphids. Post hoc comparisons of Lol⁺ versus Lol⁻, and Lol⁻ versus E⁻, were subject to Bonferroni adjustment to compensate for multiple comparisons (Sokal and Rohlf 1995).

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