Inducible defenses provided by *Neotyphodium* to *Lolium arundinacea* and *Lolium pratense*: an ecological and molecular approach

T. J. SULLIVAN, T. L. BULTMAN, J. RODSTROM, J. VANDOP, J. LIBRIZZI, C. GRAHAM, A. SIELAFF and L. FERNANDEZ
Department of Biology, Hope College
sullivan@hope.edu

Abstract

Plants have evolved a range of responses to herbivory ranging from tolerance to both inducible and constitutive chemical defenses. Previous research has shown lolines produced by *Neotyphodium coenophialum* are inducible by host damage. In this paper, we examined whether this inducibility can also be caused by damage from an invertebrate herbivore and whether this phenomenon also occurs in *N. uncinatum*. We also investigated whether changes in gene expression for *lolC*, a gene in the loline biosynthesis pathway, accompanies changes in defense response. For *N. coenophialum*, both mechanical and herbivore damage had a negative effect on subsequent aphid herbivores. For *N. uncinatum*, damage did not cause an increase in defense, although there was an increase in endophyte-free hosts. Relative expression of *lolC* varied significantly over time, and between damage types for *N. uncinatum* with herbivore damaged plants having a lower relative *lolC* -1 expression.

Keywords: *Neotyphodium*, gene expression, inducible defense, loline, *lolC*

Introduction

Many plants have evolved chemical defenses to resist herbivory, but some plants have evolved a tolerance strategy, the ability to withstand some damage without a decrease in fitness (Strauss & Agrawal 1999). Grasses, especially in resource rich environments, are usually thought to respond to herbivory via compensation (Hawkes & Sullivan 2001). The presence of *Neotyphodium* spp. endophytes may alter this strategy, however. In some host species, *Neotyphodium* spp. provide chemical defense active against both vertebrate and invertebrate herbivores (Clay & Schardl 2002; Siegel & Bush 1996). Recent work by Bultman *et al.* (2004) found that defenses provided by *N. coenophialum* to tall fescue (*Lolium arundinacea*) can be induced in response to host damage. After mechanical damage, *N. arundinacea* infected tall fescue had a significant increase in loline concentration and a significant negative effect on population growth of bird cherry-oat aphids (*Rhodopsium padi*) (Bultman *et al.* 2004). Conversely, damage to endophyte-free tall fescue caused a higher rate of regrowth and a positive effect on bird cherry-oat aphid population growth (Bultman *et al.* 2004).

Responses at the molecular level to herbivory are not well understood. DNA microarrays and mRNA differential display reverse transcription (DDRT)-PCR show changes in hundreds of genes in response to herbivory (Baldwin *et al.* 2001; Roda & Baldwin 2003) although these changes can vary depending on damage type and herbivore species (Voelckel & Baldwin 2004). *Neotyphodium* endophytes can produce several different defensive chemicals including the insecticidal lolines. The genes in the loline biosynthesis pathway have been recently identified, and expression of one of the genes, *lolC* is correlated with loline production in culture (Spiering *et al.* 2005).

In this study we examine variation in herbivore defense in response to damage in two *Neotyphodium* infected grasses, tall fescue (symbiotic with *N. coenophialum*) and meadow fescue

(*Lolium pratense*, symbiotic with *N. uncinatum*) using two approaches. First, we tested for variation in defense response using invertebrate bioassays, and second, we examined the molecular basis for the variation by quantifying variation in *lolC* expression.

Methods

Damage treatments and bioassay

Tall fescue plants (cv. Kentucky-31) were grown from seed and were damaged 6 weeks after germination either mechanically (cutting all tillers 3 cm above the soil with scissors), by fall army-worm (*Spodoptera frugiperda*) or left undamaged. Fall armyworm damage was performed by placing plants in cages (44.1 x 44.1 x 44.1 cm) and allowing the fall armyworm to feed until 40-60% of the above ground tissue was removed. Within each cage, only a single infection type was used to ensure that no plant was differentially damaged due to the presence of *Neotyphodium*. Twenty-five E+ and E- individuals were used in each treatment.

Meadow fescue individuals were generated by splitting 15 larger plants into several individuals. Damage was performed as described above and individuals derived from the original 15 plants were distributed equally between the damage treatments to control for genotype. Twenty-five E+ and E- individuals were used in each treatment.

For the tall fescue bioassay, bird cherry-oat aphid was used as the target herbivore using the youngest fully expanded leaf and the method described by Siegel *et al.* (1990). The total number of aphids present was counted after 7 days. For the meadow fescue, fall armyworm was used, but due to high mortality in the cut treatment, only individuals from the herbivore-damaged and control treatments were used. Fall armyworm were kept in individual Petri dishes and fed the youngest tissue from the plants; the development time from the 2nd to 4th instar was measured.

Quantifying *lolC* expression

150 mg of fresh tissue was taken from the base of four tillers per plant at 10 and 24 days after damage of tall fescue and 3, 6 and 22 days after damage of meadow fescue. The tissue was immediately frozen in liquid nitrogen, ground to a powder and extracted using the RNeasy kit (Qiagen). The RNA was quantified using a Hitachi U-2000 spectrophotometer. Real-time RT-PCR was performed using 100 ng total RNA and the Brilliant QRT-PCR Master Mix Kit (#600551 from Stratagene) in a SmartCycler II (Cepheid). Primers and probes for *lolC* are as described in Spiering *et al.* (2005). The cycle threshold value (Ct) was defined at the amount of fluorescence 10 standard deviations above background. The *lolC* RNA was quantified using a standard curve generated using cRNA (Fronhoffs *et al.* 2002). As each sample was a mixture of both plant and endophyte RNA, *lolC* expression was expressed as a ratio with the amount of fungal beta-tubulin (*tubB*) RNA in each sample. No reverse transcriptase controls were included for each sample to ensure there was no contaminating RNA. Each individual was run in duplicate (*N. coenophialum*) or triplicate (*N. uncinatum*) and only runs where the coefficient of variation

Figure 1 Effects of *N. coenophialum* infection and previous damage type on aphid survival on tall fescue. Error bars are ± 1 standard error.

Figure 3 Effects of time from damage and damage type on the relative expression of *lolC* in *N. coenophialum* (A) and *lolC* -1 and *lolC* -2 in *N. uncinatum* (B) over time and between damage types for *lolC* -1 in *N. uncinatum* (C). Error bars are ± 1 standard error.

Figure 2 Effects of *N. uncinatum* infection and previous damage type on fall army-worm development rate when feeding on meadow fescue. Error bars are ± 1 standard error.

was less than 5% were used. The number of valid samples were: *N. coenophialum*; 10 days post damage: E+ cut = 5; E+ herbivore = 5; E+ control = 3; at 24 days post damage (2 days post aphid assay): E+ cut = 4; E+ herbivore = 6; E+ control = 7; *N. uncinatum*; 3 days post damage: E+ cut = 7; E+ herbivore = 7; E+ control = 7; 6 days post damage: E+ cut = 7; E+ herbivore = 7; E+ control = 7; 22 days post damage: E+ cut = 6; E+ herbivore = 5; E+ control = 7).

Bioassay data were analysed using two-way ANOVA with damage type (mechanical, herbivore, or control) and endophyte infection (E+ or E-) as factors. Damage type and time from damage were used as factors in the relative *lolC* expression analysis. All data were tested for normality and Box-Cox transformed when appropriate.

Results

In tall fescue, there was a significant effect of both *N. coenophialum* infection (Fig. 1, $F_{1,144} = 144.91$, $P < 0.0001$) and the interaction between infection and damage type (Fig. 1, $F_{2,144} = 3.29$, $P = 0.04$) on the number of living *R. padi* with E+ grasses becoming more toxic to aphids after damage and E- grasses becoming more susceptible.

In meadow fescue, fall armyworm feeding on E+ grasses had an increased development time (Fig. 2, $F_{1,177} = 34.63$, $P < 0.0001$) and there was a significant interaction between damage treatment and *N. uncinatum* infection (Fig. 2, $F_{1,117} = 7.67$, $P = 0.01$). Fall armyworm also had a greater final dry mass on E+ meadow fescue ($F_{1,116} = 11.26$, $P = 0.001$).

lolC expression in *N. coenophialum* was significantly affected by both the time from damage (Fig. 3A, $F_{1,24} = 8.41$, $P = 0.008$) and the interaction between time and damage type (Fig. 3A, $F_{2,24} = 3.54$, $P = 0.045$). In the control plants, expression at 10 days was significantly less than expression at 24 days and less than the herbivore damaged plants at both time points. Expression of the two *lolC* variants in *N. uncinatum* also changed over time (Fig. 3B, *lolC*-1: $F_{2,51} = 12.41$, $P < 0.0001$; *lolC*-2: $F_{2,51} = 4.69$, $P = 0.01$) and expression of *lolC* -1 also varied by damage treatment (Fig. 3C; $F_{2,51} = 3.80$, $P = 0.03$) with the cut and herbivore treatments having a lower expression than the controls.

Discussion

The presence of *Neotyphodium* endophytes can alter its host's defensive strategy. In tall fescue, infection by *N. coenophialum* provided an inducible chemical defense against subsequent invertebrate herbivores while endophyte-free hosts became more susceptible after damage. Damage did not affect fall armyworm

development time in *N. uncinatum* infected meadow fescue, but development time did increase in herbivore-damaged, endophyte-free meadow fescue. Development time was still shorter than in the comparable infected hosts, however, suggesting that *N. uncinatum* was providing a high level of constitutive defenses, overriding any innate inducible defense in meadow fescue.

Relative expression of *lolC* varied over time for both *N. coenophialum* and *N. uncinatum*. For *N. coenophialum*, relative *lolC* expression was greater in herbivore-damaged plants compared to the control at 10 days. As *lolC* expression is correlated with loline production (Spiering *et al.* 2005), this suggests an increase in defense response in damaged plants 10 days post-damage.

Expression of both *lolC* -1 and *lolC* -2 in *N. uncinatum* varied over time, likely due to natural temporal variation in gene expression. Damage type also affected relative *lolC* -1 expression, although the control plants were higher than the herbivore damaged plants. As *N. uncinatum* infected meadow fescue had exhibited a high constitutive defense regardless of damage treatment, the lower *lolC* -1 expression may reflect a shift in resources to growth in the herbivore-damaged plants without a significant decrease in its defensive ability.

The presence of *Neotyphodium* endophytes appears to alter the defensive strategy of its hosts. In tall fescue, *N. coenophialum* provides an inducible defense for tall fescue while *N. uncinatum* provides a constitutive defense to meadow fescue.

ACKNOWLEDGEMENTS

The authors thank Drs. Martin Spiering and Christopher Schardl for their assistance in developing the quantitative RT-PCR assay. This work was funded by the National Science Foundation grant DBI-0330840 to TLB and DBI-0139035 to the Biology department at Hope College.

REFERENCES

Baldwin, I.T.; Halitschke, R.; Kessler, A.; Schittko, U. 2001. Merging molecular and ecological approaches in plant-insect

- interactions. *Current Opinion in Plant Biology* 4: 351-358.
- Bultman, T.L.; Bell, G.; Martin, W.D. 2004. A fungal endophyte mediates reversal of wound-induced resistance and constrains tolerance in a grass. *Ecology* 85: 679-685.
- Clay, K.; Schardl, C.L. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *American Naturalist* 160: S99-S127.
- Fronhoffs, S.; Totzke, G.; Stier, S.; Wernert, N.; Rothe, M.; Bruning, T.; Koch, B.; Sachinidis, A.; Vetter, H.; Ko, Y. 2002. A method for the rapid construction of cRNA standard curves in quantitative real-time reverse transcription polymerase chain reaction. *Mol Cell Probes* 16: 99-110.
- Hawkes, C.V.; Sullivan, J.J. 2001. The impact of herbivory on plants in different resource conditions: A meta-analysis. *Ecology* 82: 2045-2058.
- Roda, A.L.; Baldwin, I.T. 2003. Molecular technology reveals how the induced direct defenses of plants work. *Basic and Applied Ecology* 4: 15-26.
- Siegel, M.R.; Bush, L.P. 1996. Defensive chemicals in grass-fungal endophyte associations. pp. 81-117. In: *Phytochemical Diversity and Redundancy in Ecological Interactions*. Eds. Romero, J. T.; Saunders, J. A.; Barbosa, P., Plenum Press, New York.
- Siegel, M.R.; Latch, G.C.M.; Bush, L.P.; Fannin, F.F.; Rowan, D.D. 1990. Fungal endophyte-infected grasses - alkaloid accumulation and aphid response. *Journal of Chemical Ecology* 16: 3301-3315.
- Spiering, M.J.; Moon, C.D.; Wilkinson, H.H.; Schardl, C.L. 2005. Gene clusters for insecticidal loline alkaloids in the grass-endophytic fungus *Neotyphodium uncinatum*. *Genetics* 169: 1403-1414.
- Strauss, S.Y.; Agrawal, A.A. 1999. The ecology and evolution of plant tolerance to herbivory. *Trends in Ecology & Evolution* 14: 179-185.
- Voelckel, C.; Baldwin, I.T. 2004. Generalist and specialist lepidopteran larvae elicit different transcriptional responses in *Nicotiana attenuata*, which correlate with larval FAC profiles. *Ecology Letters* 7: 770-775.