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Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737

Dear Administrator Juarez,

This letter is to inquire whether the fungal endophyte, *Epichloë coenophiala*, with a deletion of its ergot alkaloid gene alleles, meets the definition of a regulated article as described in 7 CFR part 340.

Summary

Tall fescue, the most widely planted pasture grass in the United States, is naturally symbiotic with the seed-borne fungal endophyte, *Epichloë coenophiala*. The endophyte is important for stand longevity and maximal forage productivity of tall fescue for reasons not fully understood but apparently including enhanced drought resistance and nematode resistance. However, the most common endophyte strains produce ergot alkaloids, which are responsible for episodic toxicosis to livestock. The toxic effects of ergovaline, the pathway end product, are known. Possible effects of some of the intermediates and spur products of the pathway are unknown.

All of the required genes for production of ergot alkaloids are located in the gene cluster named *EAS*. Because *E. coenophiala* is polyploid, it has two alleles of the gene cluster, designated *EAS1* and *EAS2*. In order to eliminate all capability to make ergot alkaloids and precursors and spur products of the ergot alkaloid pathway, we used a single application of CRISPR technology to simultaneously eliminate both alleles, giving an *eas1*Δ *eas2*Δ deletion mutant.

The technique used a ribonucleoprotein preparation, plus a selectable plasmid, introduced into *E. coenophiala* by polyethyleneglycol (PEG)-mediated protoplast transformation. Analysis of the resulting *eas1*Δ *eas2*Δ mutant indicated that the plasmid had *not* integrated in the genome and, instead, the genome was altered only by CRISPR-mediated deletion of the *EAS* alleles. This possibility will be further checked by whole genome sequencing, and the proposed release will occur only if results confirm that no exogenous DNA was integrated in the genome.

Developer (Responsible Party) name and contact information, including email address.

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Taxonomic description of organism (genus, species).
Epichloë coenophiala

Description of intended phenotype.
The *eas1*Δ *eas2*Δ mutant is **unable to produce any ergot alkaloids**

Description of intended activity (movement or release).

Release. The endophyte is to be introduced into an elite breeding line of tall fescue (*Lolium arundinaceum = Schedonorus arundinaceus = Festuca arundinacea*) by meristem inoculation of seedlings. Because this fungus has no natural means of plant-to-plant transmission, it can only be maintained by transmission in seeds from systemic symbiosis (maternal line vertical transmission). Therefore, the presence of the endophyte and its mutant genotypes will be tested in symbiotic plants as well as seeds produced by those plants.

Release activities will be to increase seeds of tall fescue with the *eas1*Δ *eas2*Δ mutant and for side-by-side comparisons of field performance between tall fescue symbiotic with the mutant, tall fescue symbiotic with the wild-type parent, and tall fescue without symbiotic endophyte. If field performance is acceptable, the tall fescue lines will be used to establish paddocks to compare animal performance when grazed on the three tall fescue lines. If animal performance is good, then tall fescue seeds with the mutant will be made available for distribution to growers.

Description of intended genetic change in final product.
The final product has deletions of the two alleles *EAS1* and *EAS2*, and no exogenous DNA.

Description of vector or vector agent used to induce genetic change in the organism.

Protoplast transformation by the polyethylene glycol method.

Name of constructs

Ribonucleoprotein (Table 1, Fig. 1) and pKAES329 (Table 2).

Description of construct, including all elements in order in which they occur in construct:

A ribonucleoprotein (RNP) mixture purchased from Synthego was generated comprising two chemically modified RNA molecules and a Cas9 nuclease protein modified to include two nuclear localization signals (Cas9-2NLS). Its composition (Table 1) includes only protein and chemically modified RNA.

Table 1. Components of the ribonucleoprotein (RNP) used to delete the *EAS* alleles.

Element type	**Element name**	**Organism from which element is derived**	**Brief description of genetic element's function**
Nuclease	Cas9-2NLS	Synthego proprietory	Nuclease with nuclear localization signals
CRISPRevolution sgRNA	EAS2lpsAguide	Synthesized by Synthego	Cleavage of genome at left end of *EAS1* and *EAS2* alleles
CRISPRevolution sgRNA	EAS2lpsBguide	Synthesized by Synthego	Cleavage of genome at right end of *EAS1* and *EAS2* alleles

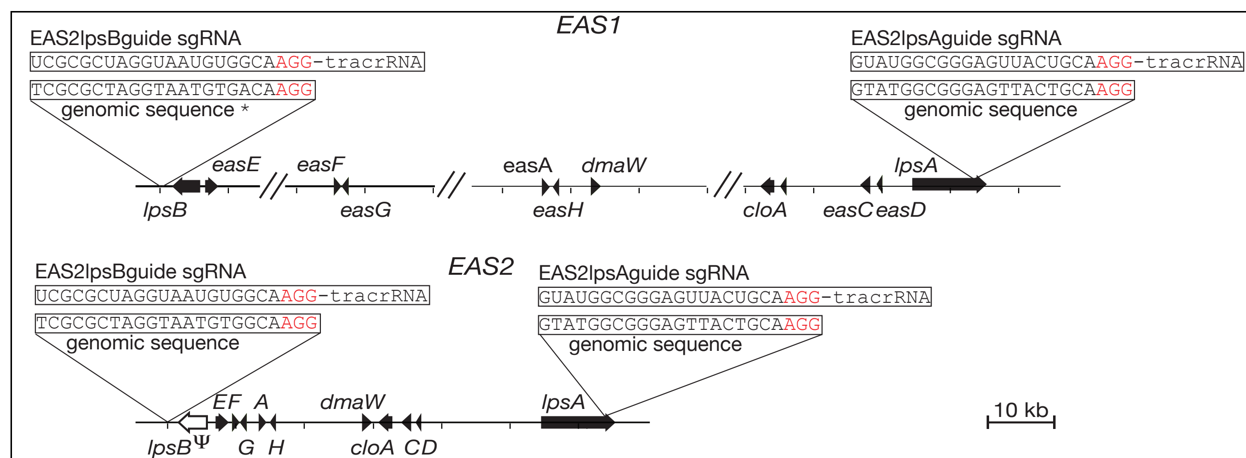

Figure 1. Maps of *EAS1* and *EAS2* alleles, and target locations of the sgRNA sequences that direct cleavage by the modified Cas9 nuclease. The tracrRNA is a 67 nt sequence that interacts with Cas9. Labeled genes are those involved in ergot alkaloid biosynthesis (some gene names are abbreviated on the *EAS2* allele map). Both alleles have been fully sequenced, but hash marks shown on the *EAS1* map indicate long stretches of noncoding sequences in that allele. The sgRNA sequences matched genomic sequence outside but near the 3' end of *lpsB* (which is a pseudogene in *EAS2*), and within but near the 3' end of *lpsA*. The mutants had the *EAS1* and *EAS2* alleles deleted between the sgRNA target sequences.

Included in the transformation mixture was plasmid pKAES329 (Table 2). The plasmid was used in a previous experiment that involved its integration and spontaneous loss due to the placement of its telomere repeat array. However, after the CRISPR mutagenesis PCR analysis of the final

product indicated that the plasmid had *not* integrated in the genome and that it had been spontaneously lost. (We will further test this by genome sequencing, described below.)

Table 2. Plasmid pKAES329 construct for selection of transient transformants, which ultimately lacked DNA from the plasmid.

Element type	**Element name**	**Organism from which element is derived**	**Brief description of genetic element's function**
Plasmid origin of replication	pUC18 ori	*Escherichia coli*	Replication in *E. coli*
Gene	*bla*	*E. coli*	Beta-lactamase gene for resistance to ampicillin
Promoter	P-*tubB*	*Epichloë festucae*	Expression of hph in *Epichloë* spp.
Phage recombination site	*loxP*	Bacteriophage P1	Cre-mediated recombination to remove hph
Gene	*hph*	*Streptomyces hygroscopicus*	Transient selection of *Epichloë* transformants
Phage recombination site	*loxP*	Bacteriophage P1	Cre-mediated recombination to remove *hph*
Telomere repeat array	Telomere	*Epichloë uncinata*	Spontaneous deletion of integrated vector
Partial gene	Partial *lpsA1*	*Epichloë coenophiala*	Homologous recombination with *eas1*

Scientific Methodology and Supporting Data

The final product will have **no inserted DNA**. PCR screens for *dmaW* and *easA* indicated that the *EAS1* and *EAS2* alleles were deleted, and a PCR screen for *hph* indicated that the selectable marker was absent from the genome. As a further test that no vector or exogenous DNA was inserted in the procedure, the genome of the mutant will be sequenced to a minimum of 50-fold coverage by Illumina HiSeq with 150-cycle reads. The data will be assembled by use of CLCBio (Genome Workbench), and the resulting contigs will be aligned by BLAST to our existing high-quality reference genome sequence of the wild-type parent. If any contigs are identified with less than 99% identity to the reference genome over their entire length, they will be selected for further investigation. Such contigs will be submitted as queries for BLAST to the entire nonredundant (nr) nucleotide sequence database of GenBank to identify their sources. Potentially some sequences may fail the initial screen because they match regions of the *E. coenophiala* genome of low complexity that give poor sequence quality. In such instances, the *E. coenophiala* genome or the *E. coenophiala* mitochondrial genome should still give a superior match to the best match in the nr database if the sequences are not derived from exogenous DNA.

Confidential Business Information
This information **Does Not Contain Confidential Business Information (CBI).**

Sincerely yours,

Christopher L. Schardl
Chair, Department of Plant Pathology