Detection of *lps*A gene in *Neotyphodium* endophytic fungi of grasses in Iran

Parisa Omoumi[1], Aghafakhr Mirlohi[2]*, Masoud Bahar[3]

[1]Department of Agricultural Biotechnology, College of Agriculture, Isfahan University of Technology, Isfahan, 84156-83111, I.R. Iran [2]Department of Agronomy and Plant Breeding, College of Agriculture, Isfahan University of Technology, Isfahan, 84156-83111, I.R. Iran [3]Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Isfahan, 84156-83111, I.R. Iran

Abstract
The *lps*A gene, a late acting gene in the biosynthetic pathway of ergovaline, a suspected causative agent for fescue toxicosis in cattle, has been cloned from *Neotyphodium lolii,* an endophytic fungus of *Lolium perenne*. In this study, a similar gene was detected in several strains of endophytic *Neotyphodium* spp. isolated from grass hosts endogenous to Iran using direct and nested-PCR assays. Except for *Bromus tomentellus*, most isolates from other hosts contained this gene. The 747-bp PCR products of the local strains had identical restriction patterns for all tested restriction enzymes. Accordingly, sequence analysis of the nested PCR product amplified from the internal segment of 747-bp band, showed 99% similarity with the corresponding region of the *lps*A gene of *N. lolii*. It therefore appears that prevalence of the *lps*A gene with its conserved nature among *Neotyphodium* isolates is mainly host dependent.
Keywords: Endophyte; *lps*A gene; Ergovaline; *Neotyphodium.*

INTRODUCTION

Endophytic fungi in the genus *Neotyphodium* confer many beneficial effects to their host plants, including resistance to pests (Clay, 1989; Clay and Cheplick, 1989), diseases (Gwinn and Gavin, 1992), grazing (Read and Camp, 1986) and environmental stresses such as soil pH fluctuations (Belesky and Fedders, 1995), and drought (Malinowski and Belesky, 2000). Although endophytes are beneficial to their host grasses, they also often produce alkaloid toxins that are harmful to livestock (Aldrich *et al*., 1993; Porter, 1995). Ergovaline is one of the ergot toxins produced by several *Neotyphodium* spp., especially those infecting tall fescue (*Festuca arundinacea*). Ergovaline consumption in livestock has been associated with poor weight gain, hormonal imbalances leading to reduced fertility, lactation and gangrene of the animal's limbs (Porter, 1995). However, a direct cause and effect relationship between ergovaline and these symptoms has not yet been demonstrated.

Ergot toxins are also produced by the ergot fungus, *Claviceps purpurea* (Tudzynski *et al*., 1999). In this fungus, the biosynthesis of ergopeptines requires the activities of two peptide synthetases, Lps1 and Lps2. The gene encoding Lps1 was first identified in *C. purpurea* (Tudzynski *et al*., 1999) and later in *Neotyphodium lolii* (in which it was named *lps*A) (Panaccione *et al*., 2001), and characterized by sequence analysis. This gene was inactivated by gene knockout method in an attempt to provide a means for identifying the roles of ergot alkaloids in the plant-fungus associations in which they occur, and for ameliorating toxicosis with which these alkaloids are associated (Panaccione *et al*., 2001).

Because of the likely significance of ergovaline to animal production, information on presence or absence of ergot alkaloid biosynthesis genes in *Neotyphodium* species of different host grasses, and sequence variability among those genes will be required. Such information will be of great importance for future employment of this symbiotic relationship in crop improvement.

*Correspondence to: **Aghafakhr Mirlohi**, Ph.D.
Tel: +98 311 3913450; Fax: +98 311 3912254
E-mail: mirlohi@cc.iut.ac.ir*

The objectives of present work were to examine the presence of the *lps*A gene and its probable sequence divergence in endophytic fungi isolated from four different grass hosts, some with high palatability and very wide distribution in the natural rangelands of Iran.

MATERIALS AND METHODS

Strains and culture conditions: Isolates of endophyte (*Neotyphodium* spp.) used in this study are listed in Table 1. Fungi were isolated from four grass species including, *Bromus tomentellus*, *F. arundinacea, F. pratensis* and *Lolium perenne* which were collected from various regions of Iran. Having confirmed the existence of *Neotyphodium* mycelia in the tissues of the samples by microscopic examination, isolations were performed either from seeds or leaf tissues of the hosts on potato dextrose agar (PDA), as described by Bacon and White (1994). All the isolates were confirmed as the *Neotyphodium* species by using specific primers, as explained by Doss *et al*. (1998).

DNA extraction: Genomic DNA was extracted from fresh mycelial mat grown in potato dextrose broth (with shaking at room temperature for 32 days). The mycelial mat was transferred to a sterile filter paper in order to remove the liquid medium. DNA was isolated by CTAB method, explained by Murray & Thompson (1980). The same method was applied in the extraction of total DNA from nodal tissues of endophyte-infected and endophyte-free grasses.

Table 1. Summary of the results from direct and nested-PCR amplification of the *lps*A gene in endophytic strains isolated from various hosts.

Plant host	Endophytic strain	Presence of 747-bp band	Presence of 414-bp band
Festuca pratensis	$FpGan_1$	+	+
F. pratensis	$FpGan_2$	+	+
F. pratensis	$FpGan_3$	+	+
F. pratensis	FpGon	-	-
F. arundinacea	FaFh	-	-
F. arundinacea	$FaTsh_1$	+	+
F. arundinacea	$FaTsh_2$	+	+
F. arundinacea	FaAl	+	+
F. arundinacea	$FaFn_1$	+	+
F. arundinacea	$FaFn_2$	+	+
F. arundinacea	FaSm	-	-
Bromus tomentellus	BtFh	-	-
B. tomentellus	BtFd	-	-
B. tomentellus	BtMh	-	-
B. tomentellus	BtAni	-	-
B. tomentellus	BtAbi	-	-
B. tomentellus	BtIn	-	-
Lolium perenne	LpAmp	+	+
L. perenne	Lp_1Prellude	+	+
L. perenne	Lp_2Prellude	+	+

PCR amplification of the *lps*A gene: For detection of *lps*A, the primer pair Lps1-F/R, were designed from the nucleotide sequences of the *lps*A gene (GenBank accession no. AF368420). The Lps1-F (5′-TTA CCgAACTggCgACAT-3′) corresponded to nucleotides 180-197 and Lps1-R (5′-ggACAC TgTACCACCACTgC-3′) was complementary to nucleotides 907-926 of the *lps*A sequence (Panaccione *et al*., 2001). This primer pair was used in the first round of PCR to direct the amplification of a 747-bp fragment from the DNA extracts. To confirm the specificity of the amplification, primer set NesLps1-F corresponding to nucleotides 356-373 and NesLps1-R complementary to nucleotides 752-769 were applied in nested-PCR to amplify a 414-bp DNA band from internal region of the 747-bp amplicon.

The amplification was carried out in a 25 µl PCR mixture containing 200 ng of template DNA, 200 µl of each dNTP, 0.4 µM of each primer, 0.75 units of Taq DNA polymerase (Roche company, Germany), and 1X PCR buffer containing 15 mM $MgCl_2$. The mixture was overlaid with a drop of mineral oil and the PCR was performed in a thermal Mastercycler (Eppendorf-Germany) programmed for an initial cycle of denaturation for 2 min at 94ºC; followed by 30 cycles of 1 min, denaturation at 94ºC, 45s of annealing at 63ºC and extension 1min at 72ºC. The final step of extension was 5 min at 72ºC. For nested-PCR, products of the primary amplification were diluted 1:30 and used as template for reamplification of the internal fragment. All sets of reactions included DNA samples from endophyte-free grasses and a control in which water was substituted for DNA.

The PCR products were separated by a 1.2% agarose gel electrophoresis, stained with ethidium bromide, and visualized with a UV transilluminator (Gel document, Vilber lourmat TCP-20-M, France).

Resterction analysis of the 747-bp fragment: For restriction analysis of the 747-bp fragment, 5 µl of each selected PCR product from the fungal isolates FaAl, $FaTsh_2$, Lp_1Prellude, $FaTsh_1$, FpGan1 and

FaFn1 were digested separately with the restriction enzymes *Pst*I, *Alu*I, *Taq*I, *Hae*III and *Sac*I (Roche, Co., Germany), according to the manufacturer instructions. The restriction products were then separated by electrophoresis on a 6% polyacrylamide gel and then stained with silver nitrate (Sambrook, 2001).

Cloning and sequencing of the 414-bp fragment: The 414-bp fragment produced by nested PCR was excised from the agarose gel and purified using the Gene Clean-III kit (Biogene-France), based on the manufacturer's instruction. The fragment was subcloned into the PGEM-T vector and transformed into *E. coli* JM109 competent cells, according to the Promega Technical Manual No.042-USA. Plasmid DNA, containing the cloned insert, was identified by blue-white screening on LB medium containing X-gall and the insert size was determined by *Eco*RI digestion and agarose gel electrophoresis. Both DNA strands of the cloned insert were sequenced by the dyedeoxy chain termination method (SEQLAB Company, Germany). Sequencing data were aligned and analyzed using the Chromas version 2.23 and searching of databases was performed by the BLASTN program.

Ergopeptine analysis: Ergopeptines were extracted from 100 mg of dried leaf clippings obtained from two *F. arundinacea* and one *F. pratensis* genotypes hosting the isolates $FaTsh_1$, $FaFn_1$ and $FpGan_1$. These extracts were then analyzed by high performance liquid chromatography (HPLC), as described by Panaccione *et al*. (2003).

RESULTS

Using the primer pair Lps1-F/R, the 747-bp target fragment (Fig. 1) was amplified from isolates of *Neotyphodium* taken from *F. pratensis* ($FPGan_1$, $FPGan_2$ and $FPGan_3$), *F. arundinacea* ($FaTsh_1$, $FaTsh_2$, FaAl, $FaFn_1$ and $FaFn_2$) and *L. perenne* (LpAmp, Lp_1Prellude and Lp_2Prellude). However, no PCR products were obtained from any of the fungal isolates belonging to *B. tomentellus* (BtFh, BtFd, BtMh, BtAni, BtAbi, BtIn). One isolate from *F. pratensis* (FPGan) and two from *F. arundinacea* (FaFh and FaSm) did not yield the intended fragment either (Table 1).

In the nested PCR assay, the primer pair NesLps1-F/R yielded a 414-bp PCR product, only in isolates which originally produced the 747-bp band (Table 1 and Fig. 2). None of these primers amplified DNA from the endophyte free test plants or control samples. Nested amplification of the 414-bp band in all the isolates produced a 747-bp band which was an additional confirmation for presence of the *lps*A gene in these isolates.

Restriction analyses of the amplified 747-bp fragment from the strains tested (FaAl, $FaTsh_2$, Lp_1Prellude, $FaTsh_1$, FpGan1 and FaFn1) using restriction enzymes *Pst*I, *Alu*I, *Taq*I, *Hae*III and *Sac*I, produced similar restriction profiles (Fig. 3).

The sequencing of the nested PCR product (414-bp band) from $FaFn_1$ and $FpGan_1$ isolates and their comparison with sequences reserved in databases, by the BLASTN program revealed that the sequence of this

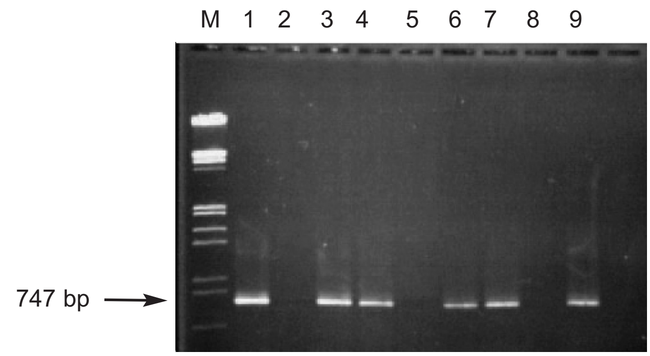

Figure 1. Direct PCR amplification of a portion of the *lps*A sequence (747-bp). M= marker, 1= *F. aundinacea* E+, 2= *F. arundinaceae* E-, 3= FaTsh1 isolate, 4= *F. pratensis* E+, 5= *F. pratensis* E-, 6= FpGan1 isolate, 7= *F. arundinacea* E+, 8= *F. arundinacea E-*, 9= FaFn1 isolate

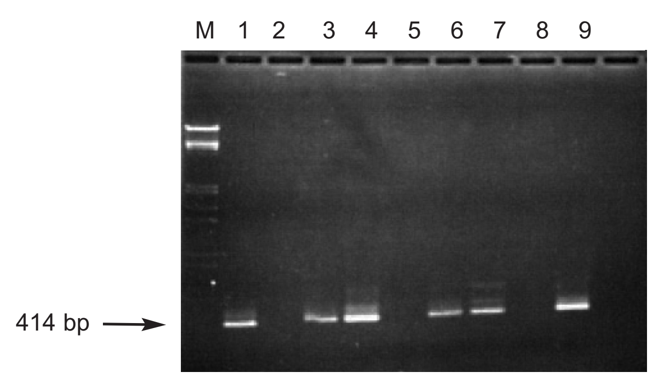

Figure 2. Nested PCR amplification of the 414-bp fragment. M= marker, 1= *F. arundinacea* E+, 2= *F. arundinacea* E-, 3= FaTsh1 isolate, 4= *F. pratensis* E+, 5= *F. pratensis* E-, 6= FpGan1 isolate, 7= *F. arundinacea* E+ 8= *F. arundinacea* E-, 9= FaFn1 isolate.

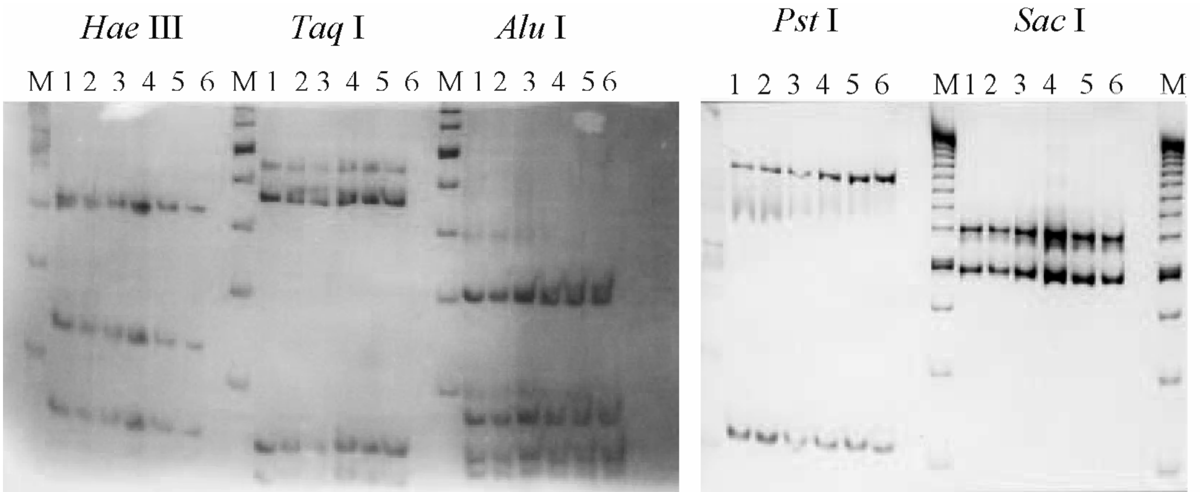

Figure 3. Restriction pattern of the 747-bp fragment from endophytic strains. 1= FpGan1, 2= FaFn1, 3= FaTsh1, 4= FaA1, 5= Lp_1Prellude and 6= FaTsh2. Products were digested with restriction enzymes *Hae* III, *Taq* I, *Alu* I, *Pst* I and *Sac*I.

fragment is identical (99%) to a portion of *lps*A gene in *N. lolii* (GenBank accession no. AF368420).

HPLC analysis showed that out of three fescue plant samples hosting endophyte isolates positive for both 747-bp and 414-bp bands, only two contained detectable quantities of ergovaline. The fescue plant samples hosting $FaTsh_1$ and $FaFn_1$ isolates contained 12.4 and 3.8 µg of ergovaline/g of plant dry weight, respectively.

DISCUSSION

Production of the 747-bp band by PCR, originating from genomic DNA of fungal isolates, provided an indication of the existence of the *lps*A gene sequence in several endophytes isolated from plants endogenous to Iran. However, this was highly host dependent and none of the isolates possessing the *lps*A gene sequence belonged to *B. tomentellus* (Table 1), which is a highly palatable perennial grass with wide geographical distribution in most arid and semi-arid regions of Iran and neighboring countries (Rechinger, 1973). This grass is present at different densities in approximately 6 million hectares of Iranian rangelands, highly infected by *Neotyphodium* and usually grazed without any symptoms of toxicosis in animals. This along with the absence of the *lps*A gene might be an indication that *Neothyphodium* isolates of this host do not produce alkaloids toxic to grazing animals, which requires further investigations.

In restriction analyses of the amplified 747-bp fragment, similar restriction profiles were produced, implying that the sequence of *lps*A gene among local endophytic isolates is conserved, and that the endophytes isolated from different hosts and geographical regions in this study are probably closely related.

The sequencing of the nested PCR product (414-bp band) and its identity to a portion of the *lps*A gene in *N. lolii* (GenBank accession no. AF368420) may further suggest the conserved nature of the *lps*A gene among isolates of *Neotyphodium* spp. from various hosts that have spread out in different geographical areas of the world.

The ergovaline quantities in fescue plant samples hosting endophyte isolates positive for both 747-bp and 414-bp bands were found to be different. This can be caused by genetic variation among the endophytic fungi for ergovaline production, or genotypic variation of plant hosts or a combination of both factors (Agee and Hill, 1994; Roylance *et al*., 1994; Ball *et al*., 1997). It seems that there is a general assumption regarding the positive correlation between the presence of the *lps*A gene and production of ergovaline(Tudzynski *et al*., 1999; Panaccione *et al*., 2001). However, the *F. pratensis* plant sample hosting the isolate $FpGan_1$ that was positive for the *lps*A gene, showing both the 747-bp and 414-bp bands during the PCR analysis, contained no detectable ergovaline. The late-acting *lps*A is a crucial gene for synthesis of ergovaline, but there are several other genes, the activities of which are required at any of the 6 or 7 steps prior to the *lps*A step (Panaccione *et al*., 2003). There could be a rearrangement in an early gene of the ergot alkaloid pathway in the $FaGan_1$ strain. Thus its failure to produce ergovaline could be due to a step earlier in the pathway, rather than that catalyzed by the *lps*A.

References

Agee CS, Hill NS (1994). Ergovaline variability in *Acremonium*-infected tall fescue due to environment and plant genotype. *Crop Sci*. 34: 221-226.

Aldrich CG, Rhodes MT, Miner JL, Kerley MS, Paterson JA. (1993). The effects of endopyte-infected tall fescue consumption and use of a dopamine antagonist on intake, digestibility, body temperature and blood constituents in sheep. *J Anim Sci*. 71: 158-163.

Bacon CW, White JF (1994). *Biotechnology of endophytic fungi of grasses*. CRC Press, Inc, Florida.

Ball OJP, Baker GM, Prestidge RA, Lauren DR (1997). Distribution and accumulation of the alkaloid peramine in *Neotyphodium lolii*-infected perennial ryegrass. *J Chem Ecol*. 23: 1419-1434.

Belesky DP, Fedders JM (1995). Tall fescue development in response to *Acremonium coenophialum* and soil acidity. *Crop Sci*. 35: 529-533.

Clay K (1989). Clavicipitaceous endophytes of grasses: their potential as biocontrol agents. *Mycol Res.* 92: 1-12.

Clay K, Cheplick GP (1989). Effect of ergot alkaloid from fungal endophyte infected grass on fall army worm. *J Chem Ecol*. 15: 169-182.

Doss RP, Clement SL, Kuy RE (1998). A PCR technique for detection of *Neotyphodium* endophytes in diverse accessions of tall fescue. *Plant Dis*. 82: 738-740.

Gwinn KD, Gavin AM (1992). Relationship between endophyte infestation level of tall fescue seed lots and *Rhizoctonia zeae* seedling disease. *Plant Dis*. 79:911-914.

Malinowski DP, Belesky DP (2000). Adaptation of endophyte infected cool-season grasses to environmental stresses: Mechanism of drought and mineral stress tolerance. *Crop Sci*. 40: 923-940.

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Res*. 8: 4321-4325.

Panaccione DG, Jahnson RD, Wang J, Young CA, Damrongkool P, Scott B, Shardl CL (2001). Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. *Proc Natl Acad Sci USA*. 98: 12820-12825.

Panaccione DG, Tapper BA, Lane GA, Davies E, Fraser K (2003). Biochemical outcome of blocking the ergot alkaloid pathway of a grass endophyte. *J Agric Food Chem*. 51: 6429-6437.

Porter JK (1995). Analysis of endophyte toxins: Fescue and other grasses toxic to livestock. *J Anim Sci*. 73: 871-880.

Read JC, Camp BJ (1986). The effect of the fungal endophyte *Acremonium coenophialum* in tall fescue on animal performance, toxicity, and stand maintenance. *Agronomy J*. 78: 848-850.

Rechinger KH (1973). *Flora Iranica*. Gramineae. By Bor, N.L. and Druck, U. PP. 30-70.

Roylance JT, Hill NS. and Agee CS (1994). Ergovaline and peramine production in endophyte-infected tall fescue: independent regulation and effects of plant and endophyte genotype. *J Chem Ecol*. 20: 2171-2183.

Sambrook J (2001). *Molecular Cloning*. Cold Spring Harbor Laboratory Press. New York.

Tudzynski P, Holter K, Correia T, Arntz C, Grammel N, Keller U (1999). Evidence for an ergot alkaloid gene cluster in *Claviceps purpurea*. *Mol Gen Genet*. 261: 133-141.