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Research Article

Development of Real-time PCR Assay Based on Hydrolysis Probe for Detection of *Epichloë* spp. and Toxic Alkaloid Synthesis Genes

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ABSTRACT

Fescues, which are widely cultivated as grasses and forages around the world, are often naturally infected with the endophyte, *Epichloë*. This fungus, transmitted through seeds, imparts resistance to drying and herbivorous insects in its host without causing any external damage, thereby contributing to the adaptation of the host to the environment and maintaining a symbiosis. However, some endophytes, such as *E. coenophialum* synthesize ergovaline or lolitrem B, which accumulate in the plant and impart anti-mammalian properties. For example, when livestock consume excessive amounts of grass containing toxic endophytes, problems associated with neuromuscular abnormalities, such as convulsions, paralysis, high fever, decreased milk production, reproductive disorders, and even death, can occur. Therefore, pre-inoculation with non-toxic endogenous fungi or management with endophyte-free grass is important in preventing damage to livestock and producing high-quality forage. To date, the diagnosis of endophytes has been mainly performed by observation under a microscope following staining, or by performing an immune blot assay using a monoclonal antibody. Recently, the polymerase chain reaction (PCR)-based molecular diagnostic method is gaining importance in the fields of agriculture, livestock, and healthcare given the method's advantages. These include faster results, with greater accuracy and sensitivity than those obtained using conventional diagnostic methods. For the diagnosis of endophytes, the nested PCR method is the only available option developed; however, it is limited by the fact that the level of toxic alkaloid synthesis cannot be estimated. Therefore, in this study, we aimed to develop a triplex real-time PCR diagnostic method that can determine the presence or absence of endophyte infection using DNA extracted from seeds within 1 h, while simultaneously detecting easD and LtmC genes, which are related to toxic alkaloid synthesis. This new method was then also applied to real field samples.

(Key words: Endophyte, Foage, Fungus, Real-time PCR)

Ⅰ. INTRODUCTION

Epichloë is a fungal endophyte classified into 43 species (Leuchtmann et al., 2014), and often naturally infects crops of the genus *Festuca-Lolium*, including tall fescue used as forage (Schardl, 1996). The majority of *Epichloë* endophytes are parasitic on plants throughout their lifetime and maintain a symbiosis by propagating through seeds without injuring or damaging the host (Schardl et al., 2004, Moon et al., 2000). Some endophytes of *Epichloë* such as *E. coenophialum*, also produce alkaloids that are toxic to predatory insects or mammals, thereby benefiting the host by increasing their survival and dominance in grasslands through increased environmental stress resistance, predator avoidance, and growth promotion (Decunta et al., 2021, Wang et al., 2020). Such a symbiotic relationship can be advantageous for the cultivation of grasses, but caution is required, as toxic alkaloids such as ergovaline and lolitrem B can accumulate in infected plants (Chujo et al., 2019). This can cause problems, such as convulsions, paralysis, high fever, decreased milk production, reproductive problems, and even death, in livestock that feed on them (Riet-Correa et al., 2013, Guerre, 2015). The infection rate depends on the region; for example it was 58-100% in the United States, 64% in the UK, and 98% on average in Finland (Clay et al., 2005). However, not all endophytes are toxic to mammals; certain strains are only toxic to herbivorous insects, while also improving drying stress resistance in infected plants. Furthermore, there was a case of commercializing these endophyte spores that are harmless to mammals in New Zealand (Hume et al., 2020). Since the quantitative evaluation of endophyte toxicity remains scarce, despite the endophytes being widespread in nature, recent studies are beginning to focus on cross-evaluating their genetic diversity, geographical characteristics, and alkaloid levels (Cagnano et al., 2019). Toxic alkaloids, such as ergovaline and lolitrem B, are

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produced by gene products expressed in approximately 11 biosynthetic gene clusters located in the genome of *Epichloë* (Chujo et al., 2019, Hettiarachchige et al., 2021). Lolitrem B is synthesized by ltmE, ltmJ, ltmP, ltmQ, ltmF, ltmC, ltmB, ltmG, ltmS, ltmM, and ltmK, while ergovaline is synthesized by lpsB, easE, easG, easA, easH, easC, easD, dmaW, cloA, and lpsA (Schardl et al., 2013). These alkaloids provide the host with increased resistance to droughts and herbivorous insects. Currently, two methods for endophyte testing are available: one using microscopy, and the other using a commercial detection kit (Agrinostics Phytoscreen kit) based on an immunoblot assay. Skilled research personnel are required for the microscopy method, with diagnosis being time-consuming and inconsistent. On the other hand, the immunoblot assay is quicker, simpler to perform, and has greater consistency than the microscopy method (Trento et al., 2007). However, this method still has limitations, including long incubation times (overnight) and variations in the results depending on the skill-level and subjectivity of the tester. Therefore, an attempt was made to develop a polymerase chain reaction (PCR)-based diagnostic method using a primer specific to *Epichloë* beta-tubulin (Dombrowski et al., 2006). However, only the presence or absence of *Epichloë* could be identified; whether the samples could generate toxic alkaloids could not be determined. Moreover, the sensitivity was relatively low due to the adoption of a single conventional PCR method. Since the synthesis of toxic alkaloids can be prevented by damaging the corresponding biosynthetic gene clusters (Panaccione et al., 2001), a technique was developed for monitoring the endophyte infection rate and toxic alkaloids by identifying not only the house-keeping genes of *Epichloë,* but also the genes involved in the biosynthesis of three alkaloids (peramine, lolines, and ergovaline, which are indole diterpenes) using multiplex PCR (Vikuk et al., 2019). However, the conventional multiplex PCR method involving electrophoresis has difficulties in interpretation due to false positives, background PCR bands from excessive DNA addition or sample contamination, and non-specific reactions. This makes its application difficult in monitoring the status of endophytes on large grasslands and monitoring endophyte contamination during seed breeding, production, and distribution as well as analysis of whether imported and exported grasses are infected with endophytes. Therefore, in this study, we aimed to develop a real-time PCR-based diagnostic method that extracts DNA from the seeds and tissues of tall fescue and detects the presence of *Epichloë* infections and alkaloid synthesis genes.

Ⅱ. MATERIALS AND METHODS

1. Designing of real-time PCR primer and collection of template nucleic acid sequence information

For the development of a real-time PCR method to specifically detect *Epichloë* spp., the sequences of the beta-actin gene (a house-keeping gene of *Epichloë* spp.), and the LtmC and easD genes (key genes from the gene cluster involved in the biosynthesis of alkaloids toxic to mammals), registered in NCBI GenBank, were obtained (Table 1). These sequences were then aligned and visualized using MAFFT (Katoh and Standley, 2013) to search for conserved sites (Fig. 1, Table 1). Thereafter, the quantitative PCR (qPCR) primer was designed according to a previous study (Bustin and Huggett, 2017) after analyzing the melting temperature (Tm) value and hairpin structure (Table 2).

2. Genomic DNA extraction

DNA was extracted from seeds and plant tissues of five cultivars of tall fescue (*Festuca arundinacea*) (Greenmaster, Purmi, Greenmaster2ho, Greenmaster3ho, and Greenmaster4ho) developed by the Grassland and Forages Division of the National Institute of Animal Science. Ten grains or 50 mg of tissue per sample were placed in a 3.0 mm pre-filled ceramic bead tube (Invirustech, Prep-HD30, Gwang-ju, Korea) for crushing. For DNA extraction, the lysis buffer included in the Clear-S™ Quick DNA extraction kit (Invirustech, IVT3002) was added according to the product manual before the sample was crushed twice using a homogenizer (Bead Ruptor Elite; Omni International, GA, USA) for 30 s at 4.5 ms^{-1}. The extracted DNA was then measured using an epoch microplate reader (BioTek, VT, USA) and stored at -65 ℃ until PCR amplification.

3. Real-time and conventional PCRs

The extracted DNA (50 ng) was amplified using EzAmp™ qPCR 2X Master Mix (Elpisbiotech, EBT-1805, Daejeon, Korea). The detection mix was prepared by mixing the tub2, easD, and

Table 1. Consensus sequences of Tub2, easD, and LtmC from *Epichloë* spp.

Gene	Sequences (5'à3')
Tub2	ATGCGTGAGATTGTAAGTTCAACCTCTCTGTTTGTCTTGGGGACCCCCTCCTCGACGCGTTCCGGTGTTGAGX XCCCCTGATTTCGTACCCCXXXXXGCCGAGCCCGGCCACGAMGXTGCACXXXXXXXGCCCAACGRACXAGT CGTGATGAGAGGCGGACCGAGXACXXXXXXAAAATTAATGAATGCGGTAXTTCGAGAACTGXTAGCTGACC XXXXXXXXXXYYTYTYTTYCCCTCTAGGTTCATCTTCAAACCGGTCAGTGCGTAAXXGTGACAAATYCGCCG ACCTCGAACGACAGGCACAAAYAXXRCATGXAAAAACTCACATTSATTTGGGCAGGGTAACCAAATTGGTG CTGCTTTCTGGCAGACCATCTCTGGCGAGCACGGCCTCGACAGCAATGGTGTGTACAATGGTACCTCCGAGC TCCAGCTCGAGCGTATGAGTGTCTACXXXXXXXXXXXXXXXXXXXXXXXXTTCAACGAGGTAAGTCTTCAT AATCTXXXAAAGTCTCCATTGAGCTACATANNNNCCGCCCYGGAGATGRGACGGAAAGAGAACGAAAXXXX XXXXXGAAAAAGTGTXXTATCATGCTMATCYATGTGACAGGCTTCTGGCAACAAGTATGTTCCTCGCGCTGT CCTCGTCGATCTCGAGCCTGGTACCATGGATGCAGTCCGTGCCGGTCCCTTCGGTCAGCTTTTCCGTCCTGAC AACTTCGTCTTCGGTCAGTCTGGTGCTGGCAACAACTGGGCCAAGGGT**CACTACACTGAGGGTGCTG**AGCTG GTTGACCAGGTCCTCGACGTTGTGCGTCGCGAGGCCGAAGGCTGTGAC**TGCCTCCAGGG**<u>**TTTCCAGATCACC**</u> <u>**CACTCGCTTGG**</u>TGGTGGTACCGGTGCCG**GTATGGGTACATTGTTGATCTCC**AAGATCCGTGAGGAGTTCCCCG ACCGGATGATGGCYACTTTYTCCGTCGTYCCCTCTCCCAAGGTCTCTGACACCGTTGTCGAGCCCTACAACGC CACTCTCTCCGTCCACCAGCTTGTCGAGAACTCGGACGAAACGTTCTGTATCGATAATGAGGCCTTGTACGAT ATCTGCATGCGTACTCTTAAGCTGTCYAACCCSTCRTACGGTGATCTGAACTACCTGGTCTCCGCTGTCATGTC TGGCGTCACCACCTGCCTGCGTTTCCCTGGTCAGCTGAACTCTGACCTGCGKAAGCTGGCCGTCAACATGGTT CCTTTCCCTCGTCTGCACTTCTTCATGGTCGGCTTCGCTCCCCTGACCAGCCGTGGCGCCCACTCTTTCCGCGC TGTTAGCGTTCCTGAGCTTACYCAGCAAATGTTCGACCCCAAGAACATGATGGCTGCTTCTGATTTCAGAAAT GGTCGCTACCTGACCTGCTCYGCCATTTTGTGAGXTGAAATTGXXACAACCCTAACTTGAXGAGTCXXGTGT CGCAGTAACTAACTGGGAACXAAACAGCCGTGGCAAGGTCGCTATGAAGGAGGTCGAGGACCAGATGCGTA ACGTGCAGAACAAGAACTCTTCCTACTTCGTCGAGTGGATCCCCAACAACATCCAGACTGCTCTCTGCGCCAT CCCTCCCCGTGGCCTCAAGATGTCTTCTACCTTTATCGGTAACTCCACCTCCATCCAGGAGCTCTTCAAGCGG GTTGGCGAGCAGTTCACGGCCATGTTCCGTCGCAAGGCTTTCTTGCATTGGTACACTGGCGAGGGTATGGAC GAGATGGAGTTCACTGAGGCTGAGTCTAACATGAACGATCTTGTTTCCGAATACCAGCAGTACCAGGATGCT GGTATTGATGAGGAAGAAGAGGAGTATGAGGAGGAGGCACCTGTTGACGAGCCTCTGGAGTAA
easD	TCATGGCGCACYCGCTCCAACGTTAATGTTACTTCCGTATACCGGCCRGGAGTCT**TCGCTCAACAGCCAGACAAT** **GGTCCGCGCGACATCTTCAGGT**TCAACMAYCGAGARGCCCTGGCTCTCGTACAGAGACTTGACTTCAGCCAGGGAC TTTGCATTAGGCTCAAATTGAGGTAGCAGTGGTGTGTTTGTAACTCCTGCTCTACATGCGTGAGCCAATGCTTGCTTG TCTTTGAWTRGTGCCSGRGRTCNTTTTCTTACC**TGGCGAGACACAGTTAACCC**GAATGCCCACCGGGAATACGTCCG TTGCGACACATGCAGTGAAGTAAGCACAGGCTCCTTTAGACGTGCCATAKGCAAARACGTCCGGTACATGTGAAAA AGCAGCTATACTGGCCACGTTGACGATACTGCGGTTGCCAGCGGGCAAGTCCTTCATAGCTACCACTTCGGCACGAG TGCAGTAAAAAACGCCGTCTAGRTTAACTTTCATGATGCGACTCCARTCTTCGTCTGTCTCGGCAAGAATTKTCGGCG ACTGGCGTAATCCCGCRCCCTGAGCTATGCCGGCGACGTTAGCCGCGCCATGAAGGTCACAGTGTTCAGAAACGATG TCTYCCAACCACTTGTTGACRGCACTGGATGAGGYGACATCAACAACTGTGCACTGGACYARGGTCGACTGATTGAT TTCGTGGACRGACTTCTTCAGGCTGTCAAAGTTRGTGCTTRYAACATCGGCTACGCAGATGACGGCMGCATCRCGGT KGGCAAGCAGTCGGCAGGTCGCGGCGCCTATGCCAGATGCACCCCCGGTAATGGCRAAAATCTTGGATGATACCGA TGCCAT
LtmC	TCATTTTGTANAGTGCATNTGTTCTTTTCGTCCCCAGACGGCCACCAAGTTCTCTAGACCCTGNCCTGCCTCCTGG AGCGTTTTGAGACACGTGTTTTTAACACTAGGTGACTCNAACGCTTGGAGTGCCCGTTTAATGTCGCCTTGATTTYGG CTCTGAAACGCCTTCCTTATCTGCGGCTCAGTATGTTTGTCATTAAGAGCAACCACAACAGGAAAGGACAATTCTCT GTTGCGTAGGTCTTCTGCAACAGTGCCTTTGTTHAAAGCGTACTCTTCTGAGTATATGTTCTTGCAATCATTTTGCAA TTGTGCGTACCAGCTACCATTSCATTGAATTAGTTAAGCGACCTTGCCGCGGAATTATGAAACGAGCGXNXXAAAAA AAAAAAAAWTACATACCCAAATCGRCCTAACAGRTCGTCTGATTGATGACCTCCTTGGTTCAAAAGCCTCCCAAGG AGCACAAAAAGTGTGCCTGTCTTCAGCAGAGCCATWTTTTTGTAC**GTCAACAGGCTTTCTTCACCA**TRGGATT**CGAA** **AGATCGCAAACCGTCTCTTCGCC**ARACCAAAGACATGTCTTGTCCCTCGAGTATHAGTTCTAGTGCTCTCAGAAGTTC AATTCCAAGGACAGGTTGTTCTTTCATTGCTCTATTAATGACCTTTGTGAGAACGAAGTATGCTCGATTG**GCAGTTTC** **GCATGATCCGTA**TAGCAAGTGAGCCGTAGTGTGATTTTTCCGCTTRGGGCTGTGGTCGCAAATATCGTCAACCATAA TTAGACAAAGATGGACCGCATCCATGATATCCAGGATCAAAGAATRTTTCTTRGGATATTYAKSTTTCAATGTTGGA TGAAGAAAATCCACGAGGGGAGAGAAATGATTATGCCCATAGAGACTAATCACATAAGAGTATGGACAGTCAGGA ATCTTGCCATTGCTGCCGCCAATACCTTCGTGAGAGGTACGTTCATAGCCATAGCCATAATGATCAATGGCTTGAGA AACGACAGATTKGTAATTGATTGTGTACTTTACYAGATACCCGAGCGTAAATGCAAACAAGAGGGCCGCAATNTCG ATGNCCGCANGGCGAGCCACNAGCCATGCTCNAGATGTCAT

* MAFFT sequence alignment is in 5'→3' orientation. Primer sequences are located under red box (forward primer), yellow box (internal oligo), and blue box (reverse primer).

ltmC-specific primers and probes (Table 1), with 10 nM of primer and 2.5 nM of probe. For PCR amplification, heat activation was performed at 95 °C for 5 min using qTower3 (Analytik Jena, Jena, Germany), and the denaturation was repeated 40 times in total by heating at 95 °C for 15 s and at 60 °C for 60 s for primer annealing and extension, respectively. The fluorescence signal was read at each cycle. To compare sensitivity and confirm infection, conventional PCR was used to amplify the tub2 gene by nested PCR, which was then electrophoresed on a 1.5% agarose TBE gel at 130 V for 40 min to confirm the PCR band

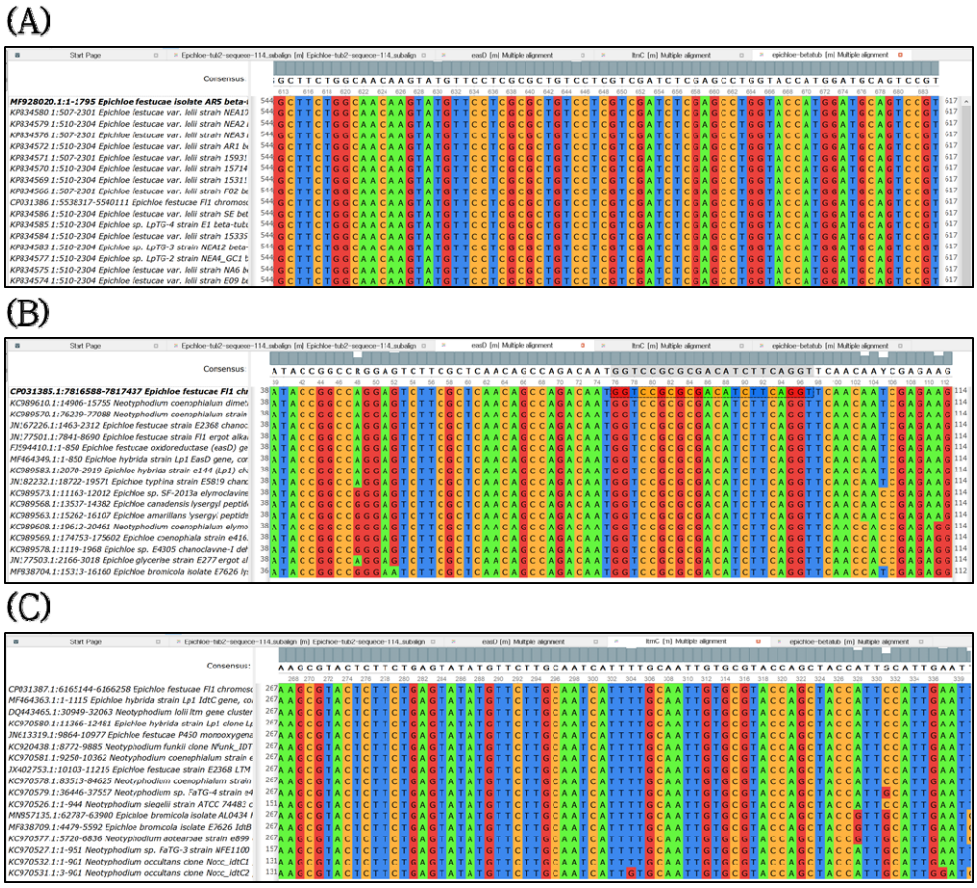

Fig. 1. Alignment and visualization of *Epichloë* spp. genes. (A) Beta-tubulin (Tub2), (B) easD, and (C) LtmC genes.

Table 2. Primers and probes for specific real-time PCR for Tub2, easD, and LtmC genes

Gene	Primer designation	Primer sequence (5′-3′)	Modification
Tub2	Ep_Tub2-Fw Ep_Tub2-Rev Ep_Tub2-IO	CACTACACTGAGGGTGCTG GGAGATCAACAATGTACCCATAC TTTCCAGATCACCCACTCGCTTGG	- - FAM-BHQ1
easD	Ep_easD-Fw Ep_easD-Rev Ep_easD-IO	TCGCTCAACAGCCAGACAAT GGGTTAACTGTGTCTCGCCA GGTCCGCGCGACATCTTCAGGT	- - CY5-BHQ1
LtmC	Ep_LtmC-Fw Ep_LtmC-Rev Ep_LtmC-IO	GTCAACAGGCTTTCTTCACCA TACGGATCATGCGAAACTGC CGAAAGATCGCAAACCGTCTCTTCGCC	- - ROX-BHQ2

(Dombrowski et al., 2006).

Ⅲ. RESULTS AND DISCUSSION

A total of 227 *Epichloë* genes, including Tub2, easD, and LtmC, were obtained from NCBI GenBank to design an *Epichloë*-specific PCR primer. All sequences entered into the reverse complement were modified to fit the ORF. Thereafter, multiple sequence alignment was performed with MAFFT v7.487 and was visualized using UGENE before the consensus sequence was secured. After checking the Tm value, amplicon size, and primer secondary structure from the obtained consensus, primers and internal oligos were selected for production. Subsequently, the genomic DNA was extracted from seeds and plant tissue samples for each tall fescue cultivar, which was measured using a spectrophotometer. The extracted DNA was quantified and diluted to not exceed 500 ng per PCR, and following PCR

Table 3. DNA yields, purity (260/280), and PCR amplification of *Epichloë* genes from tall fescue

No.	Host	Cultivar	Sample	260/280	ng/μL	Tub2	ltmC	easD	cvPCR
1	*F. arundinacea*	Fawn	Seed	1.78	112.13	-	-	-	-
2	*F. arundinacea*	Green master	Seed	1.798	118.47	-	-	-	-
3	*F. arundinacea*	Purmi	Seed	2.122	36.243	+	+	+	+
4	*F. arundinacea*	Green master no.2	Seed	1.997	97.123	-	-	-	-
5	*F. arundinacea*	Green master no.3	Seed	2.008	60.592	-	-	-	-
6	*F. arundinacea*	Green master no.4	Seed	1.92	60.071	-	-	-	-
7	*F. arundinacea*	Fawn	Blade	1.794	224.616	-	-	-	-
8	*F. arundinacea*	Green master no.2	Blade	1.902	319.509	-	-	-	-
9	*F. arundinacea*	Green master no.3	Blade	1.89	185.288	-	-	-	-
10	*F. arundinacea*	Green master no.4	Blade	1.668	231.913	-	-	-	-
P.C	Infected seed from Neotyphodium immunoblot detection kit					+	+	+	+

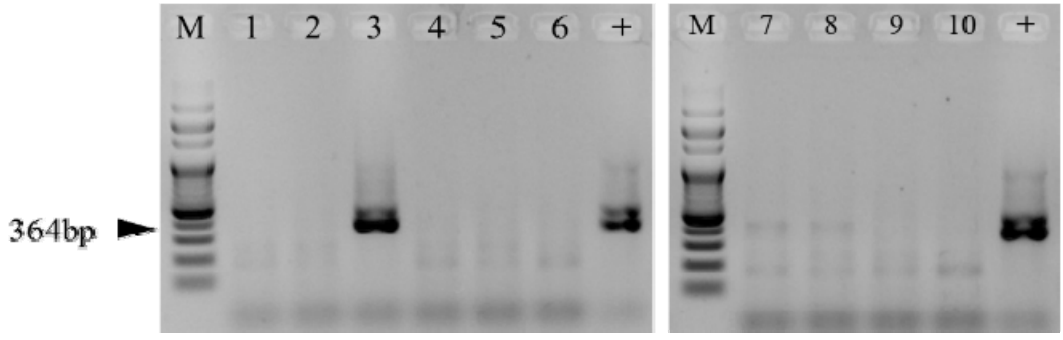

Fig. 2. Amplification of PCR amplicon using DNA extracted from seed and tissue samples from tall fescue. M, Enzynomic 100 bp DNA ladder; (1-6) seed of *F. arundinacea*; (7-10) leaf blade of *F. arundinacea*; (+) infected seed from tall fescue.

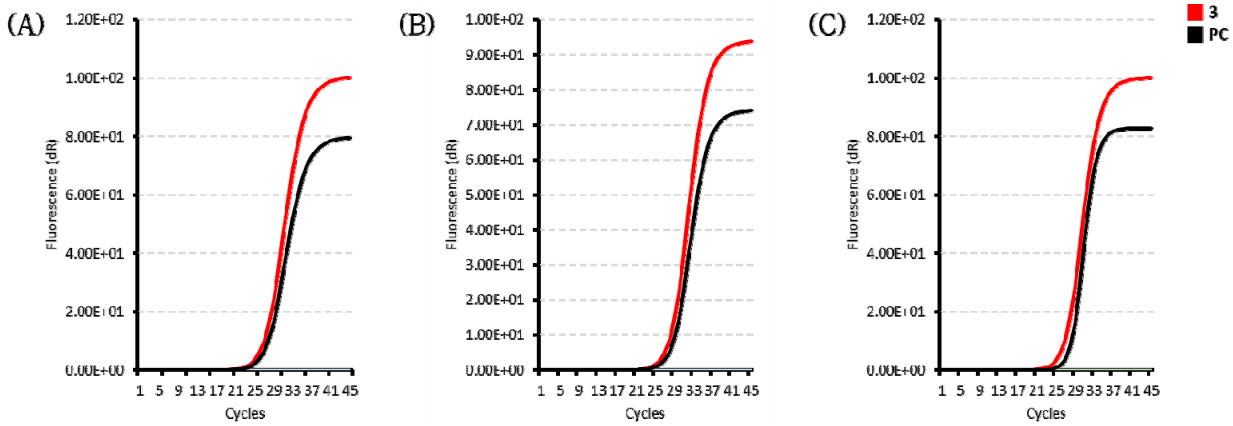

Fig. 3. Amplification curves for (A) Tub2, (B) ltmC, and (C) easD genes of *Epichloë* spp. Blue line, Sample 3, Purmi seed

analysis, *Epichloë* spp. were detected in some samples. In the *Epichloë* Tub2-specific nested PCR, a product of the expected size, at approximately 364 bp, was confirmed (Fig. 2). Multiplex PCR confirmed the presence of *Epichloë* infection and detected the toxic alkaloid genes LtmC and easD, as detected in the nested PCR (Fig. 3). Based on the sequences of known *Epichloë* spp. genes, real-time PCR primers and probes were designed, and PCR conditions were established. Based on the references,

infection was compared and analyzed using nested PCR primer sets, and the sensitivity and specificity were confirmed to be consistent. We also noted that the new diagnostic method developed through this research does not require skilled research technicians, unlike microscopy- or immune blot-based diagnostic methods. In addition, the limitations of the nested PCR method, such as high cross-contamination despite increased sensitivity and accuracy, were improved. Importantly, the existing diagnostic method could only confirm the presence of endophytes, whereas our multiplex real-time PCR method was able to detect endophyte infection and the toxic alkaloid genes. As this multiplex real-time PCR method indirectly confirms whether the infected endophyte is toxic to livestock, it has the potential to be utilized as a basic monitoring tool for more precise and safe grass production.

Ⅳ. CONCLUSION

Epichloë, a type of fungus, is well known as an endophyte of major plants and herbs, such as tall fescue. Some *Epichloë* spp. have positive functions, such as increasing the drying stress resistance of host plants through alkaloid synthesis and accumulation, or improving grass survival owing to increased avoidance by herbivorous insects. However, the absence of endophytes is more advantageous in the use of high-quality grasslands for livestock production as alkaloids that are toxic to mammals, such as lolitrem B or ergovaline, may accumulate in the plants. In conclusion, a multiplex real-time PCR method was developed in this study that could detect easD and LtmC, which are toxic alkaloid synthesis genes, as well as Tub2, which is a house-keeping gene. The test results were consistent with those obtained by the nested PCR method, with a detection accuracy of up to 0.1 ng or less. *Epichloë* spp.-specific gene amplification through real-time PCR could not only diagnose endophyte infection in grasses but also determine whether the imported seeds were infected. The findings of this study may aid in the risk assessment of endophytes.

Ⅴ. ACKNOWLEDGEMENTS

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