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PCR-mediated detection of *Ustilago esculenta* in wateroat (*Zizania latifolia*) by ribosomal internal transcribed spacer sequences

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ABSTRACT

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PCR-mediated detection of *Ustilago esculenta* in wateroat (*Zizania latifolia* Turcz.) tissue have been developed. The internal transcribed spacer (ITS) region of the ribosomal DNA from the representative *isolate* of *U. esculenta* was amplified by PCR using conserved ITS primers. The entire ITS region was cloned into a pCR™II vector, and sequenced. Two PCR primers, Ue10 and Ue11, were designed based on ITS 2 sequences, and tested for the detection of *U. esculenta* in wateroat tissue. The primer pair was subsequently shown to amplify predicted-size fragments from the DNA of all *U. esculenta* isolated from wateroats which were collected from the major growth area in Taiwan. On the contrary, this primer pair failed to amplified any fragments from the DNA extracted from other tested pathogenic fungi. Using this primer pair, it was successful to rapidly detect *U. esculenta* in DNA extracted from as few as 10 mg of diseased wateroat tissue. The amplification products could be detected in the DNA only from growth tip and sheath of diseased plant, but not in the leaves of diseased plants. The detection limit of the primer pair was tested and shown to amplify DNA at the pg level.

Key words : internal transcribed spacer (ITS), ribosomal DNA (rDNA), *Ustilago esculenta*, wateroat (*Zizania latifolia* Turcz.), PCR diagnosis

INTRODUCTION

Wateroat (*Zizania latifoolia* Turcz.), widely used as a vegetable in Taiwan, is a fungus gall induced by *Ustilago esculenta* P. Henn. It is characterized as heat and wet tolerant, stable and high yielding, less insect and diseases, and known as no chemical residual vegetable [(7)]. Cultivars of the wateroat are normally divided into green, red, and white according to the color of the outer skin of the gall. The green and white cultivars are planted in early spring and forms galls from March to October. Red cultivar is planted later and harvested in September. Flowering plants have no commercial values due to no gall formation. *U. esculenta* within the plant is the dikaryotic mycelium phase, which grows intercellularly and intracellularly, and is distributed systematically in the stem tissue of wateroat. Due to great yield loss causing by flowering wateroat, the availability of improved diagnostic techniques for the rapid and accurate identification of *U. esculenta* becomes very important in planting strategies.

The polymerase chain reaction (PCR), a highly sensitive and specific tool for the amplification of nucleic acids, was largely applied to the diagnosis of plant pathogenic fungi [(1,2,8,9,11,14)]. The high sensitivity of PCR enables quick and accurate detection of only a few fungal cells in infected plant tissues, often well before visible disease symptoms are apparent. The sensitivity of PCR- mediated detection and its applicability for detection in minimal amount of tissue and complex environments, such as roots or soil, make it an ideal diagnostic technique. The technique not only required less time to perform but was also more sensitive than other molecular detection methods. Thus, pathogen diagnostics

based on PCR technology are likely to become an important aspect of modern integrated pest management practices in agriculture.

PCR has been used for detection of a number of fungal pathogens based on the internal transcribed spacer (ITS) regions of ribosonmal DNA (rDNA) [5]. Fungal nuclear rDNA and the ITS regions offer several advantages for molecular diagnosis. They generally exists high copy numbers, allowing for detection in preparations where DNA is in very low concentrations or where pathogen DNA is a small fraction of the total DNA, e.g., a mixture of fungal and plant DNA. Moreover, rDNA possess conserved as well as variable sequences and can be amplified and sequenced with universal primers based on their conserved sequences [3,5]. Considerably greater sequence variation is also found in the ITS regions between the rDNA genes since different coding and noncoding regions of rDNA evolve at different rates [10,17]. The ITS regions evolve more quickly than the genes themselves and may vary among species within a genus, allowing the development of PCR primers that uniquely amplify a given species. The ITS regions of rDNA have been shown to be generally conserved at the species level but variable in higher taxa [3].

The objectives of the present study were (1) to sequence the ITS region 2 of *U. esculenta* ; (2) to construct specific oligonucleotide primers based on the ITS sequences; and (3) to use the primers to establish the PCR-mediated assay for the detection of *U. esculenta* in wateroat tissue.

MATERIALS AND METHODS

Fungal isolates

Twenty-two isolates of *Ustilago* spp., which were collected from different geographical origins in Taiwan, used in this study are listed in Table 1. These isolates were grown on potato dextrose agar (PDA) slant at 26℃, and stored in a 10℃ incubator. For long-term preservation, the sporidia suspension was stored in 30 % aqueous glycerol and kept at -80 ℃. All other plant pathogenic fungi used in this study were isolated by and maintained in our lab.

DNA extraction

The sporidia of *Ustilago* isolates were inoculated into Czpek-Dox broth amended 0.1 % yeast extract, and grown at 26℃ for three to four days. The culture suspension was centrifuged, and lyophilized. Genomic DNA from each isolate was extracted using the method of Yoon et al. [19]. DNA extraction from diseased or healthy wateroat tissue followed the method described by Edwards et al. [4]. Ten mg of fresh plant tissue was merceated, and vortexed in 400μl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS). The extracts were centrifuged at 12,000 rpm for 1 min and 300μl of the supernatant was mixed with 300μl isopropanol and left at room temperature for 10 min. Following centrifugation at 12,000 rpm for 10 min, the pellet was air dried and dissolved in 50μl TE buffer (1 mM Tris-HCl, 0.1 mM EDTA pH 8.0).

Table 1. Isolates of *Ustilago* spp. used in this study.

Isolate	Species	Host (cultivar)	Location
Ue070	*U. esculenta*	wateroat (green)	Nantow
Ue94-3	*U. esculenta*	wateroat (green)	Nantow
Ue119	*U. esculenta*	wateroat (green)	Chiayi
Ue124	*U. esculenta*	wateroat (green)	Tainan
Ue125	*U. esculenta*	wateroat (green)	Taichung
Ue130	*U. esculenta*	wateroat (green)	Changhua
Ue132	*U. esculenta*	wateroat (green)	Chiayi
Ue136	*U. esculenta*	wateroat (green)	Chiayi
Ue137	*U. esculenta*	wateroat (green)	Nantow
Ue152	*U. esculenta*	wateroat (green)	Nantow
Ue000	*U. esculenta*	wateroat (early-grown)	Nantow
Ue123	*U. esculenta*	wateroat (red)	Nantow
Ue128	*U. esculenta*	wateroat (red)	Ilan
Ue135	*U. esculenta*	wateroat (mottle)	Ilan
Ue122	*U. esculenta*	wateroat (white)	Nantow
Ue153	*U. esculenta*	wateroat (unknown)	Taichung
Uz90-1	*U. maydis*	corn	Nantow
Us96-1	*U. scitaminea*	sugarcane	Taichung
Uc97	*U. cynodontis*	bermuda-grass	Taichung
Ua102	*U. avenae*	oat	Taichung
U111	*U. coicis*	*Coix lachrymajobi*	Taichung
U142	*Ustilago sp.*	awn	Taichung

PCR amplification condition

PCR amplifications were performed in 25μl volumes containing 1μM of each primer, 0.2 mM of each of the four deoxyribonucleotixes, 1 unit of Primezyme (Biometra Inc., Tampa, FL) in 1X PCR buffer. Temperature parameters were 94℃ for DNA denaturation, 45 sec for the first cycle and 15 sec for the remaining cycles; 52℃ for 15 sec for primer annealing, and 72℃ for 15 sec for primer extension. The total number of amplification cycles was 30 plus a final extension of 2 min at 72℃. The reactions were run using the Perkin-Elmer Cetus 9600 thermal cycler (Perkin- Elmer Co., Norwalk, Conn.). The amplified products were separated by

gel electrophoresis in 2 % agarose. Gels were stained in ethidium bromide and visualized under 260 nm ultraviolet (UV) light.

Sequencing of the ITS2 region and primer synthesis. The ITS region 2 from representative isolate (Ue070) of *U. esculenta* was cloned and sequenced. The product of the amplification reaction was used directly for ligation into a plasmid vector (pCR^{TM}II) using TA Cloning Kit (Invitrogene Co., Carlsbad, CA) and transformed into competent *Escherichia coli* cells according to the manufacturer's instructions. DNA sequence was determined using Klenow Phototope Sequencing Kit (New England Biolabs Inc., Beverly, MA) and universal primers for the vector. The specific primers were designed by PC-Gene software based on their positions along the ITS 2 region. These primers were synthesized in the Applied Biosystems Division, Perkin-Elmer Taiwan Co.

Southern blotting

DNA was transferred to nylon membrane (Micron Separations Inc., Westboro, MA) by Southern blotting as described by Sambrook et al. [12]. Hybridization was done at 68℃ followed the method provided by the manufacturer. Digoxigenin was detected by chemilminescence, using 3-(2'spiroadamantane)-4-mehoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane (Lumigen-PPD, Boehringer Mannheim, Gaithesburg, MD) with 3-hr of film exposure.

RESULTS

Characteristics of the ITS region of *Ustilago* spp.

The fungal DNA extracted from six isolates of *U. esculenta* and six isolates of different *Ustilago* species were amplified using the universal primers ITS 1 and ITS 4 [15]. The PCR product were obtained for all 12 isolates, and the size of the amplification fragments were around 600 bp based on agarose gel electrophoresis (Fig. 1). Genomic DNA from *U. esculenta* isolates resulted in a single 600-bp fragment, whereas amplification product of other *Ustilago* species showed variable fragment sizes.

The ITS 2 sequence of *U. esculenta* and design of primers

The length of the entire ITS 2 region of *U. esculenta* was 254 bp based on DNA sequencing (Fig. 2). Comparison of the sequence with the PC-Gene sequence database confirmed that the amplified DNA sequences were similar to the ITS 2 regions of other fungi, such as *Leptosphaeria maculans* and *Neurospora crassa* (data not shown). The DNA sequence did not show homology with any reported DNA sequences of PC-Gene system. Based on the 254-bp sequence, two primers, Ue10 and Ue11, were designed. The sequences of primers Ue10 and Ue11 were 5' GCGGGTTTCAGAAGCACTCC 3', and 5' TTGGCGGATCGGTAATGAGG 3', respectively.

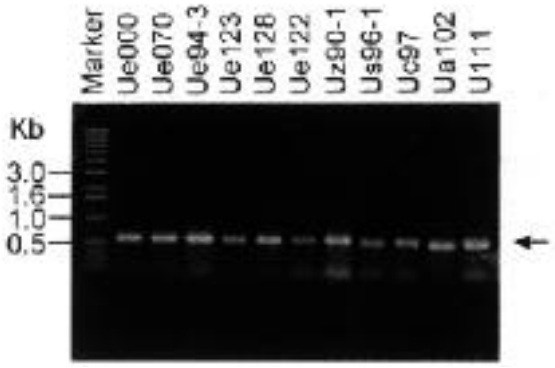

Fig. 1. Agarose gel electrophoresis of the polymerase chain reaction-amplified products from the DNA of *Ustilago* species using universal primers ITS1 and ITS4.

Test for primer specificity

Using primers Ue10 and Ue11, the PCR products were amplified from the DNA of 16 *U. esculenta* isolates from different cultivars of wateroat in Taiwan. The amplified products were 208 bp in length, which was the expected DNA fragment size based on the original DNA sequence (Fig. 3). There were no differences in the location of several endonuclease recognition sites in the fragments amplified from the different isolates (data not shown). However, this primer pair failed to amplify any fragments from the DNA extracted from other plant pathogenic fungi, including *Sclerotinia sclerotium, Collectotrichum gloeosporides, C. musae, Penicillium digitatum, Fusarium solani, Alternaria brassicica* (Fig. 4).

Detection of *U. esculenta* in wateroat

The ability of the Ue10 and Ue11 primers to detect *U. esculenta* in diseased plants was tested by amplifying total DNA extracted from greenhouse-grown wateroat tissues. The primer pair successfully and consistently produced a 208-bp PCR product from diseased plants (Fig. 5). On the other hand, no amplification fragments were observed with DNA extracted from healthy wateroat tissues which were not infected by *U. esculenta*. Fig. 6 illustrated the amplification

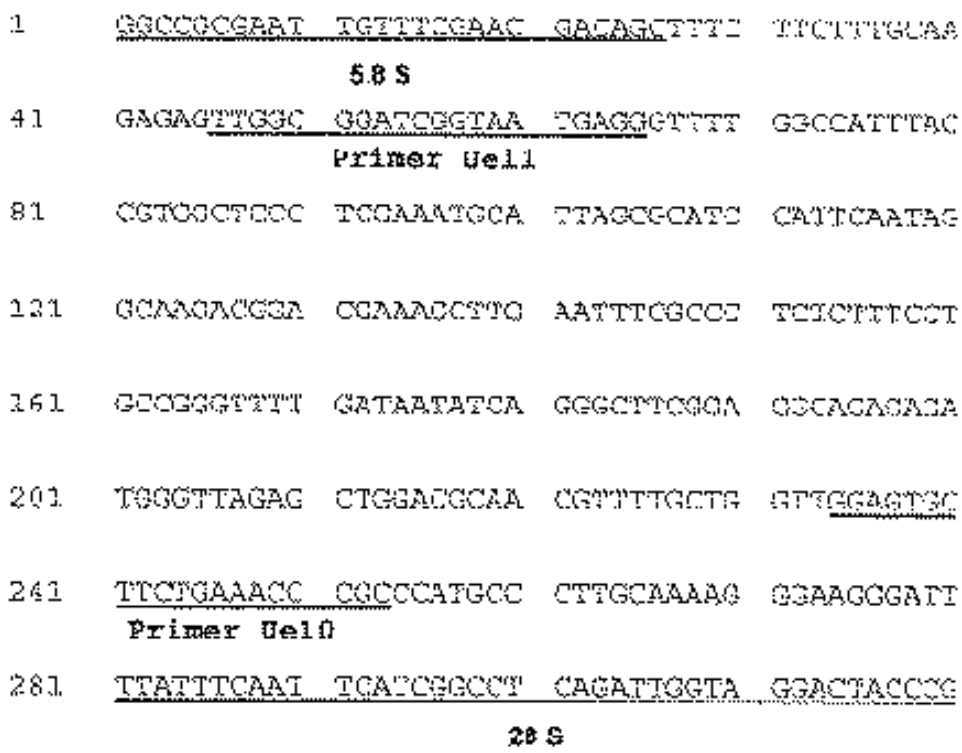

Fig. 2. DNA sequence of the internal transcribed spacer (ITS) region 2 of *Ustilago esculenta*.

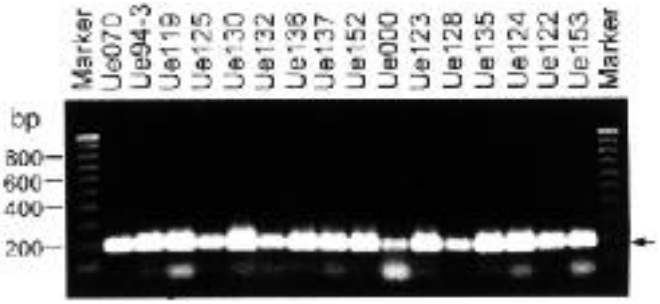

Fig. 3. Agarose gel electrophoresis of the polymerase chain reaction-amplified products from the DNA of different isolates of *Ustilago esculenta* using primers Ue10 and Ue11. Expected-size (208 bp) fragment is observed in all lanes.

products of the DNA extracted from different part of diseased and healthy wateroat, and the DNA extracted from *U. esculenta* culture. The amplification products could be detected in the DNA only from growth tip and sheath of diseased plant, but not in the leaves of diseased plants. The identity of the amplification fragment was confirmed by hybridization with the digoxigenin-labeled portion of the *U. esculenta* ITS 2 region (Fig. 6).

PCR detection limits

Sensitivity of the PCR-mediated detection using primers Ue10 and Ue11 was tested using 10-fold serial dilution of *U. esculenta* genomic DNA. The amplification product was obtained with as little as 10 pg of DNA as a template, and also observed with 1 pg of DNA (Fig. 7). However, there was no observable amplification detected at the fg level of DNA (Fig. 7).

DISCUSSION

PCR has been used successfully to specifically detect a number of fungal plant pathogens in pure culture and infected plant tissue. High sensitivity of the PCR assay has also been shown to be capable of detecting a single molecule of template DNA [8]. In the present study, the results have shown that PCR amplification using primers derived from the DNA sequence of the ITS 2 region of ribosomal DNA is an ideal tool for detection of *U. esculenta* in wateroat tissue. The consistent DNA amplification of a 208-bp fragment from different parts of infected plants with the primers Ue10 and Ue11 showed that the primer pair could be used to detect *U. esculenta* in wateroat tissue even prior to the appearance of symptoms. Moreover, the small-scale DNA extraction procedure was demonstrated to be suitable for PCR detection from wateroat tissue, since it would not affect the growth of wateroat. In addition to a high degree of specificity and

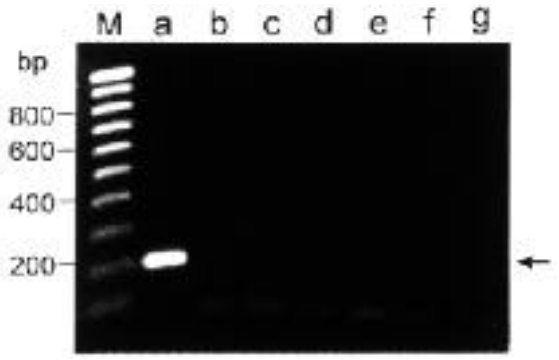

Fig. 4. Agarose gel electrophoresis of the polymerase chain reaction-amplified products from the DNA of *Ustilago esculenta* and other pathogenic fungi using primers Ue10 and Ue11. Lane a: *U. esculenta* Ue 070; lane b: *Sclerotinia sclerotiorum*; lane c: *Collectotrichum gloeosporioides*; lane d: *Collectotrichum musae*; lane e: *Penicillium digitatum*; lane f: *Fusarium solani*; lane g: *Alternaria brassicae*.

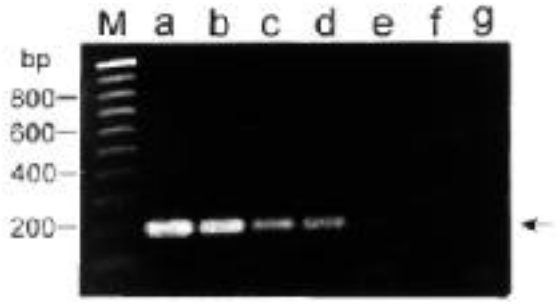

Fig. 5. Agarose gel electrophoresis of the polymerase chain reaction-amplified products using primers Ue10 and Ue11. Lane a: Ue 070 total DNA; lane b: DNA from wateroat gall tissue; lane c and d: DNA from outer and inner shoot tissue of diseased wateroat, respectively; lane e and f: DNA from outer and inner shoot tissue of healthy wateroat, respectively; lane g: negative control.

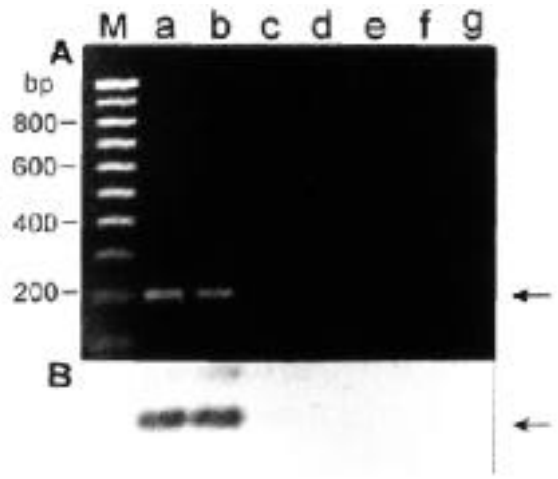

Fig. 6. PCR-mediated detection of *Ustilago esculenta* in wateroat tissue. (A) Agarose gel electrophoresis of the PCR amplification products using the DNA extracted from different part tissue of wateroat. Lane a, b, and c: DNA from growth tip, outer shoot, and leave of diseased wateroat, repectively; lane d, e, and f: DNA from growth tip, outer shoot, and leave of healthy wateroat, respectively; lane g: negative control. (B) Southern hybridization using digoxigenin labeled ITS1 and ITS 4 amplification product as a probe.

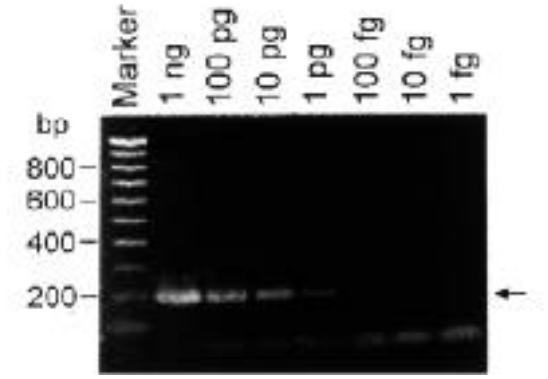

Fig. 7. Agarose gel electrophoresis of the polymerase chain reaction-amplified products from amplification of 1 ng to 1 fg of *Ustilago esculenta* DNA using primers Ue10 and Ue11.

sensitivity, the present PCR-mediated diagnosis assay was also rapid, and results could be routinely obtained from genomic DNA within one working day.

PCR amplification of fungal DNA in a crude DNA extraction from wateroat tissue allowed for the detection of the presumably small amounts of the fungus. We have shown that amplification with primers Ue10 and Ue11 could detect as little as 1 pg of *U. esculenta* DNA (Fig. 7). Still, the sensitivity of PCR does require extra precautions to avoid contamination. Control amplifications of uninoculated plant tissue and extration buffer lacking DNA were always included to monitor contamination. The specificity of this detection method would be even more important when working with plants infected with *U. esculenta* in the field. In our study, common plant pathogenic fungi were tested negative with this detection method. However, amplification products could be found while other *Ustilago* spp. DNA was tested with this primer pair, suggesting that these primers could not be able to distinguish between *Ustilago* spp. (data not shown). Other different primer pairs will further be designed and evaluated so that the primers may be more specific to *U. esculenta*.

The ability to detect *U. esculenta* in wateroat tissue provided a method to study fungal infection of the host.

Previous histological studies of wateroat tissue have shown that *U. esculenta* distributed systematically throughout the stem tissue, but were not found in leaves and root tissues [18]. The results that amplification products were obtained from the DNA of wateroat growth tip, and no amplification were found from the DNA of leaves and healthy plant tissue (Fig. 6) in our study coincided with these observations. Employment of the additional Southern transfer hybridization with the digoxigenin-labeled portions of the ITS 2 region could further proved the accuracy of the amplification results. We have also shown that *U. esculenta* is present in the sheath of infected wateroat plants, even when there were no outward signs of its presence (Fig. 5). Since the sensitivity of microscopic evaluation was low and the procedure was destructive, it was not possible to monitor symptom development in the plants that were examined. The method described in the present study will aid the advanced assay of infection process of *U. esculenta* in wateroat tissue.

The ITS regions was selected for primer development because: 1) they occur as multiple copies within the fungal DNA; 2) they tend to be similar within but variable between fungal species; and 3) species-specific oligonucleotides for other plant pathogens have been identified within the ITS regions [6,10]. Sequence analyses of the ITS region in *U. esculenta* provide a means for developing species-specific oligonucleotide primer to selectively amplify DNA of smut fungi. The specificity of this PCR assay is based on divergent sequences of the ITS region of rDNA genes that exhibit considerable sequences for differentiation of closely related fungal plant pathogen has proved successful. Although rDNA sequences are conserved, it appears that the ITS region has enough variation so that primers can be identified for diagnosis of fungal plant pathogens. The ITS region is also useful for studying genetic relationships among plant pathogenic fungi. Despite the high conservation between functional rDNA coding sequences, the nontranscribed and transcribed spacer sequences are usually less well conserved and are thus suitable as target sequences for the detection of recent evolutionary divergence [13,15]. Therefore, detailed sequence analyses of the ITS regions may be used for estimating phylogenetic relationships among pathogenic fungi. In order to understand the evolutionary relationship of smut fungi in Taiwan, sequencing the ITS regions of different *Ustilago* spp. is ongoing, and the result will be further published elsewhere.

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摘　要

陳瑞祥[1,3]、曾德賜[2]. 1999. 利用內轉錄區間序列及PCR偵測茭白筍中之黑穗病菌. 植病會刊8:149-156.
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本研究主要目的為篩選對茭白黑穗病菌具有專一性及敏感性之引子對，配合聚合酵素連鎖反應 (PCR) 增幅技術，開發於田間快速準確偵測茭白黑穗病菌之方法。首先利用過去相關真菌核糖體DNA研究中所發展所得之通用性引子，自茭白黑穗病菌DNA增幅獲得其核糖體DNA上之內轉錄區間 (ITS) 序列。經解序反應並與已發表的真菌核糖體DNA序列加以比對後，利用PC-Gene軟體設計偵測用引子對Ue10 及Ue11。利用此引子對皆可自測試的16株茭白黑穗病菌DNA中獲得一208 bp 之增幅產物，而其他常見的植物病原真菌則無類似反應。進一步應用此引子對與茭白植株組織萃取所得 DNA 反應發現，可於生長點及葉鞘部位偵測到黑穗病菌，但在葉片則未見黑穗病菌的分布；而其偵測靈敏度經測試可達pg程度。本研究結果顯示此引子對的專一性及高靈敏性可實際應用於偵測田間感染茭白植株之黑穗病菌。

關鍵詞：茭白黑穗病菌、聚合酵素連鎖反應 (PCR)、內轉錄區間序列、核糖體DNA、偵測